THE EFFECT OF LAPAROSCOPY ON IMPLANTATION, DISSEMINATION AND GROWTH OF INTRA ABDOMINAL MALIGNANCY

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by

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TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The work presented in this thesis documents the establishment of a reproducible model of carcinoma implanted into the abdominal wall of an immunocompetent Dark Agouti rat, which has been used to study the relationship between laparoscopy and the development of port site metastases.

At the commencement of this work, the factors leading to the development of port site metastases following laparoscopic surgery of tumours had not been elucidated. The aims were to examine aspects of laparoscopic surgery and their association with the development of port site metastases, as follows:

- surgical technique, environment and instrumentation;
- dissemination, implantation and growth characteristics of tumour cells and
- alterations in the host environment and responses.

The initial work showed an increased incidence of metastases to laparoscopic wounds following laparoscopic laceration of the abdominal wall tumour. The distribution pattern of metastases suggested factors inherent in the laparoscopic environment contributed. The elimination of CO_2 pneumoperitoneum, by gasless laparoscopy, lowered the metastatic incidence to that which occurred by laparotomy. CO_2 may play a crucial role in the development of this phenomena.

An alternative model using free cells injected into the peritoneum demonstrated that CO_2 laparoscopy leads to increased peritoneal distribution and growth of tumour, when compared to gasless laparoscopy or laparotomy. Subsequent studies investigated the capacity of circulating CO_2 to transport cells, by using radio labelled free cells introduced into the peritoneal cavity. These studies revealed viable tumour cells were transported outside the peritoneal cavity by large gas leaks. Laparoscopic procedures increased peritoneal dispersion of cells when compared to laparotomy. The possible immune and metabolic effects of CO_2 on the peritoneal environment are discussed.

Further studies conducted revealed that the tumour bearing state or altered peritoneal environment influenced the tumour spread to wounds and the peritoneum. When macrophage activation following CO_2 pneumoperitoneum was investigated, the preliminary data indicates CO_2 may have a suppressive effect on peritoneal macrophage activation. In a subsequent study immunohistochemistry revealed that macrophage infiltration of laparoscopic wounds was significantly decreased following CO_2 insufflation. Surface pH measurement showed significant acidosis of the peritoneum following CO_2 insufflation. These findings suggest that CO_2 significantly changes the immune and metabolic environment of the wound and peritoneum.

The development of port site metastases appears to be multifactorial and further studies are needed to clarify issues raised in this work. Until the mechanisms of port site metastases are fully understood, this work gives support to the contention that laparoscopic surgery for malignancy should be conducted only within the context of clinical trials.

DECLARATION

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University and that to the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the text.

I give consent to this copy of my thesis when deposited in the university library being available for loan and photocopying.

George Mathew

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PREFACE

During the course of the work presented in this thesis, the findings have been presented at national scientific meetings and some manuscripts published (reprints in the Appendix) or accepted for publication. These are listed below:

Mathew G, Watson DI, Rofe AM, Baigrie CF, Ellis T & Jamieson GG. (1996) Wound metastases following laparoscopic and open surgery for abdominal cancer in a rat model. *Br J Surg* 83: 1087-1090.

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Mathew G, Watson DI, Ellis T, DeYoung N, Rofe AM & Jamieson GG. (1997) The effect of laparoscopy on the movement of tumour cells and metastasis to surgical wounds. *Surg Endosc* (In Press).

LIST OF ABBREVIATIONS

DA	Dark Agouti
DAMA	Dark Agouti mammary adenocarcinoma
CO_2	carbon dioxide
IL	interleukin
FNA	fine needle aspiration
IP	intraperitoneal
PBS	phosphate buffered saline
^{51}Cr	radio-isotopic Chromium 51
TNF-α	tumour necrosis factor - alpha
LPS	lipopolysaccharide
ANOVA	analysis of variance

CHAPTER 1 INTRODUCTION

1.1. Historical Overview

The art of surgery has witnessed a number of technological advances in the last two decades. Surgeons have incorporated developments in the fields of electronics, optics and video imaging into the routine practice of surgery. This technology transfer marks the beginning of minimally invasive surgery or laparoscopic surgery as it is known today. The word 'laparoscopy' is derived from the Greek words *lapara,* meaning "the soft part of the body between ribs, hip, flank and loin" and *skopein,* which means "to look at or survey" *(Churchill's Illustrated Medical Dictionary).* Surgeons have always held a fascination for learning techniques to explore body cavities without making large incisions to gain access. Surgeons down the centuries have always recognised the importance of examining internal compartments of the body, especially the abdominal cavity, to diagnose the source and nature of diseases.

An Arab physician, Abu Kasim of Cordoba has been credited with the first recorded attempt in history of examining the cervix by reflected light around 9th century AD (as quoted by Filipi *et al,* 1991). The earliest report of attempts to examine the body cavity is attributed to Phillipe Bozzini in 1805, who constructed a crude apparatus to visualise the urethra, using candle light reflected by mirrors (as quoted by Lau *et al,* 1997). Desormeaux in 1865, developed the first serviceable endoscope to inspect the bladder. He used a kerosene lamp as the light source and a lens to narrow the beam achieving a brighter spot (as quoted by Kurtzer, 1994). A more contemporary style endoscope was produced by Max Nitze, a German urologist in 1879 (as quoted by Lau *et al,* 1997). Following the invention of incandescent light by Edison, the light bulb was added at the distal end of the cystoscope scope by Newman in 1883 (as quoted by Dameword, 1992).

The first published report of examining the peritoneal cavity with an endoscope was by Jacobaeus, a Swedish surgeon in 1901, where he used a cystoscope to examine the peritoneal cavity in humans, without distending it with air (as quoted by Cuschieri & Buess, 1992). In 1902, Georg Kelling in Germany reported using a cystoscope to examine the peritoneal cavity of dogs, after distending it with air (as quoted by Marguiles & Shabot, 1993). It is difficult to determine which of them had the original idea, but is most likely that both the developments occurred independently at about the same time.

The early techniques used air, that was pumped in manually by a syringe, to create a pneumoperitoneum. Gotez in 1921, developed a needle for insufflation (as quoted by Lau *et al,* 1997). Veress, a Hungarian, developed a modified spring loaded needle for the safe introduction of gas in 1938 (as quoted by Decker, 1946). This spring loaded needle had an inner stylet that automatically converted the sharp cutting edge to a rounded end, incorporating a side hole. This needle, with refinements, is still being used today and is named after him. Zollikoffer, in 1926, proposed the use of CO_2 as the insufflating gas to substitute for air (as quoted by Lau *et al,* 1997). Fevers, in 1933, demonstrated that CO_2 can be used safely as an alternative to air in patients (as quoted by Cuschieri & Buess, 1992).

One of the most significant landmarks in the development of this technique was the development of the rod lens system by Hopkins, a British physicist, in 1952 (Cuschieri & Buess, 1992). The Hopkin's rod lens system remains the basis for the modern rigid endoscope used in laparoscopy, although it has undergone refinements.

Jacobaeus performed the first thoracoscopic procedure in 1925, when he cauterised and divided adhesions between the visceral and parietal pleura in a patient with pulmonary tuberculosis (as quoted by Cuschieri & Buess, 1992). It was Jacobaeus who first coined the word *laparothorakoskopie* when he published his description of the inspection of human peritoneal and thoracic cavities. The development of

thoracoscopic surgery, as we know today, is from the pioneering work of Wittmoser over a period of three decades from 1951 (as quoted by Cuschieri & Buess, 1992).

Kurt Semm, of Kiel University in Germany, is undoubtedly the father of modern laparoscopic surgery. Kurt Semm in the 1960s, developed the first automatic insufflator that monitored abdominal pressure and the rate of flow of gas (Semm, 1967). He also developed the multipuncture laparoscopic system. His pioneering work resulted in a series of technological advances that led to complicated laparoscopic procedures. He contributed enormously to the development of laparoscopic equipment such as: laparoscopic dissectors, forceps, tissue morcellator and endoloop applicator and many more, which are now routinely used in endoscopic surgery (Semm, 1989). Most of the gynaecological laparoscopic procedures currently practised were developed by him. He performed the first laparoscopic appendectomy in 1981 (Semm, 1983).

General surgeons were later participants in the race to acquire these technical skills and adopt operative laparoscopy. An early advocate of the use of laparoscopic techniques was George Berci (1973). He pioneered the use of laparoscopy for diagnostic problems and trauma. Paul Sugarbaker (1976) and Alfred Cuschieri (1978) were the other early advocates. The visualisation of the abdominal cavity was restricted in the earlier years. Developments in the field of video imaging, electronics and miniaturisation after the arrival of the silicon chip, gave a tremendous boost to the cascade of developments in laparoscopic surgery. The development of the computer chip video camera in 1986, attachable to the laparoscope, improved the clarity and field of vision tremendously (Filipi *et al,* 1991). Surgeons were now able to embark on performing surgical procedures hitherto considered not possible. The enthusiasm generated amongst the general surgeons following the success of laparoscopic removal of the gall bladder has snowballed all over the world. This has also triggered a revolutionary change in the practice of gastrointestinal surgery.

The important people associated with the landmark developments in laparoscopic surgery are as follows:

1981	Semm	laparoscopic appendectomy (Semm, 1983)
1987	Mouret	laparoscopic cholecystectomy (Filipi *et al,* 1991)
1989	Buess	endoscopic oesophagectomy (Becker *et al,* 1993)
1990	Katkhouda	truncal vagotomy and seromyotomy (Katkhouda & Mouiel, 1991)
1991	Cuschieri	abdominal cardio myotomy (Shimi *et al,* 1991) thoracoscopic oesophageal myotomy (Shimi *et al,* 1992)

The success of laparoscopic biliary tract surgery has made the minimally invasive option gain immense popularity. This has also propelled surgeons all over the world to use this surgical technique in the management of other intra abdominal disorders. Laparoscopic appendectomy has become popular ever since Semm demonstrated its feasibility in 1981 (Semm, 1983).

Dubois in 1989 demonstrated the feasibility of laparoscopic highly selective vagotomy (Dubois, 1994). Katkhouda performed the first selective vagotomy and now many surgeons are performing this procedure on a regular basis. The most popular anti ulcer laparoscopic treatment being a posterior truncal vagotomy and an anterior seromyotomy (Katkhouda & Mouiel, 1991).

In 1991, Dallemagne *et al* demonstrated that laparoscopic Nissen fundoplication could be performed, giving as good results as open surgery. This approach is fast replacing the open approach.

Delaitre reported the first splenectomy that was performed laparoscopically in 1991 and in many centres this approach is being increasingly used for the removal of the spleen (Delaitre & Maignien, 1991). Schlinkert *et al* (1991) performed a

laparoscopically-assisted right hemicolectomy in 1991. Goh *et al* (1992) performed laparoscopic partial gastrectomy in 1992.

Diagnostic laparoscopy for the staging of malignancy has become an accepted practice, as it avoids the morbidity of an abdominal incision. While the advantage of visual examination and ability to biopsy suspicious tissue is present (Fernandez-del-Castillo & Warshaw, 1993), there is a concern that some lesions which are hidden from direct view will be missed, as the advantage of palpating for the texture and consistency of tissues is lost (Slim *et al,* 1996).

The applications of a laparoscopic approach to operations are increasing every day and it is beyond the scope of this review to list all the operations that can be carried out through the laparoscope. Only the passage of time and careful research will determine if these applications are advantageous to patient care, when compared to conventional surgery.

1.2. Physiological and Immunological Effects of Laparoscopy.

As laparoscopy is beginning to be practised widely, the physiological effects surrounding laparoscopy are beginning to be understood. The most important pathophysiological changes during laparoscopy are related to pressure effects and the use of CO_2 (Baxter & O'Dwyer, 1995). There are concerns about the effects of large volumes of carbon dioxide (CO_2) being introduced into the peritoneal cavity under raised pressures. These concerns include the effect of CO_2 on the blood biochemistry and gases, the effect on pulmonary function and the incidence of embolic phenomena (Baxter & O'Dwyer, 1995). Carbon dioxide is a readily soluble gas, which diffuses readily across membranes including the peritoneum (Monagle *et al,* 1993).

Laparoscopy requires the establishment of pneumoperitoneum in order to provide adequate surgical exposure and to maintain the operating space. Insufflation of carbon dioxide into the peritoneal cavity, however, can affect several homeostatic systems leading to alterations in acid base balance, blood gases, cardiovascular and pulmonary physiology (Milheiro *et al,* 1996). Although these changes may be well tolerated by healthy individuals, they may increase physiological stress in patients with pre-existing conditions, placing them at increased risk for perioperative complications (McDermott *et al,* 1995). Changes in pulmonary physiology during laparoscopy are almost entirely mechanical and primarily due to the pneumoperitoneum, with lesser effects arising from a change in the patient's position (Safran & Orlando, 1994). Peritoneal insufflation causes an increase in both the intra abdominal volume and intra abdominal pressure, both of which impede diaphragmatic excursion. As a result of this, airway pressure rises, whereas the pulmonary compliance and vital capacity falls. Ventilation perfusion mismatch and pulmonary shunting contribute to a decrease in arterial oxygenation (Milheiro *et al,* 1996).

The CO_2 pneumoperitoneum causes a significant elevation in serum CO_2. This hypercarbia is primarily due to trans peritoneal absorption of CO_2. Increase in pCO_2 to 55 - 70 mmHg may cause elevation of the heart rate, systolic blood pressure, central venous pressure, cardiac output and left ventricular stroke volume, with a decrease in peripheral vascular resistance (Safran & Orlando, 1994).

Mild hypercarbia (pCO_2 of 45 - 50 mmHg) however, appears to have little impact on haemodynamic function. Carboperitoneum in healthy patients rarely exceeds this limit (Ramussen *et al,* 1997). Although most patients develop only mild hypercarbia, patients with cardiopulmonary dysfunction and hypermetabolic states, may be at an increased risk for developing moderate to severe hypercarbia and acidemia, with concomitant cardiovascular responses (Safran & Orlando, 1994).

Subcutaneous emphysema, extra peritoneal insufflation and increased duration of insufflation are associated with a greater degree of CO_2 absorption during laparoscopic pelvic operations (Wolf *et al,* 1995).

The use of carbon dioxide insufflation during laparoscopic cholecystectomy in American Society of Anaesthetists (ASA) classification I and II patients, resulted in small statistically significant, but clinically insignificant elevation of the end tidal carbon dioxide levels. This was also a reliable reflection of the changes in arterial CO_2 tension (Monagle *et al,* 1993). It has been recognised that laparoscopic port placements, especially in upper abdominal surgery, result in a significant decrease in pulmonary function when pre operative and post operative measurements are compared (Mohsen *et al,* 1996). It has also been demonstrated that laparoscopic cholecystectomy decreased the pre operative pulmonary function by a quarter, while the open procedure resulted in halving it (Peters *et al,* 1993).

Toxic levels of carbon monoxide (CO) can occur when eletrocautry is used in the presence of CO_2 pneumoperitoneum and is absorbed into the circulation. In a group

of patients undergoing laparoscopic cholecystectomy who were studied, CO levels remained within normal limits, probably due to the short duration of intraperitoneal smoke present in the pneumoperitoneum (Esper *et al,* 1994).

A profound but reversible reduction in renal cortical perfusion occurs which seems to be entirely due to the increased intra-abdominal pressure (Chiu *et al,* 1995; Hunter, 1995) and is likely to be mediated by elevated anti diuretic hormone (ADH) and aldosterone levels, which have been postulated to be released in response to the stretch of the peritoneum (Mikami *et al,* 1996). This change could be significant in patients with renal transplant and altered renal function (Koivusalo *et al,* 1996).

Carbon dioxide pneumoperitoneum may lead to mechanical compression of splanchnic vein and the presence of CO_2 may cause splanchnic vasoconstriction (Dwerryhouse *et al,* 1995; Schilling *et al,* 1997). CO_2 pneumoperitoneum of long duration may induce intestinal ischaemia and hepatic or pancreatic dysfunction (Ishizaki *et al,* 1993).

Animal studies have demonstrated that there is a progressive decrease in femoral blood flow with increasing intra abdominal pressure due to the pneumoperitoneum. The major decrease in venous outflow occurred at pressures commonly employed for laparoscopic surgery. Release in pneumoperitoneum resulted in large increases in venous out flow (Jorgensen *et al,* 1994a). This finding was confirmed by doppler studies on a group of patients undergoing laparoscopic cholecystectomy. Baseline peak femoral blood flow velocity decreased significantly and the femoral vein diameter increased with the creation of pneumoperitoneum. This effect has implications for the potential development of deep vein thrombosis (DVT) and pulmonary embolus (PE) (Jorgensen *et al,* 1994b).

In a series of patients who underwent biliary tract surgery laparoscopically, no increase in venous CO_2 or air embolism was detected (Landercasper *et al,* 1993).

When core temperature was studied in a group of patients who underwent laparoscopic cholecystectomy, there was a statistically significant, but clinically insignificant, fall in temperature (Monagle *et al,* 1993).

There was no significant difference in hormonal profile, but urinary vanillyl mandelic acid (VMA) was increased indicating increased catecholamine release (Griffith *et al,* 1995; Ortega *et al,* 1996), when patients undergoing laparoscopic cholecystectomy were compared to those having open surgery. In the same group of patients there was a diminished acute phase response (Griffith *et al,* 1995).

It has been postulated that translocation of bacteria across the bowel may occur during laparoscopy causing contamination of the pneumoperitoneum (Oshodi *et al,* 1996).

The small size of the access wounds has led some to postulate that the metabolic response to surgical stress may be reduced. However, interleukin 6 (IL-6) levels were found to be no different in a randomised study comparing the open and laparoscopic operation (Ellstrom *et al,* 1996; Fukushima *et al,* 1996).

Wound pain is reduced after laparoscopic surgery compared to that after open surgical access to perform the same surgical procedure (McMahon *et al,* 1994a). The degree of stretching of the intra abdominal cavity is an important source of post operative pain (Wallace *et al,* 1997).

Laparoscopic abdominal surgery seems to offer some advantage in recovery of gastrointestinal motility compared to laparotomy (Hotokezaka *et al,* 1996) and this may enable early oral nutrition and mobilisation (Bardram *et al,* 1995).

There is some evidence to suggest that laparoscopic cholecystectomy causes less depression of cell mediated immunity than open surgery (Griffith *et al,* 1995). Mealy *et al* (1992), recorded that laparoscopic cholecystectomy stimulates a significant stress response and the cortisol and catecholamine response is similar to that seen in patients after open cholecystectomy. In an experimental study on mice using delayed hypersensitivity testing following laparoscopic and open manipulation, it was demonstrated that the delayed hypersensitivity was preserved after laparoscopic surgery (Trokel *et al,* 1994). In a comparative evaluation of tissue trauma after laparoscopic and abdominal hysterectomy, results showed that the post operative interleukin IL-6 levels were the same in both groups (Ellstrom *et al,* 1996). It is contended that the proposed advantages of laparoscopic surgery may be attributable to factors other than reduced tissue trauma inflicted upon the patient during the operation (Ellstrom *et al,* 1996; Fukushima *et al,* 1996). Thus the assumption, that laparoscopic surgery results in less operative stress is only partly substantiated with the evidence available so far (Ortega *et al,* 1996).

1.3. Laparoscopic Port Site Metastases

Following the introduction of laparoscopic cholecystectomy (reviewed by Filipi *et al,* 1991) there has been a surge of enthusiasm and a rapid acceptance of laparoscopic techniques in surgery. Thus there has been a rapid expansion in the application of minimally invasive techniques to abdominal and thoracic surgery. There are now reports in the published literature of laparoscopic colectomy (Schlinkert, 1991), gastrectomy (Goh *et al,* 1992), pancreatectomy (Cuschieri *et al,* 1996; Salky & Edye, 1996), nephrectomy (Clayman *et al,* 1991), splenectomy (Delaitre & Maignien, 1991), adrenelectomy (Costantino *et al,* 1993) and liver resection (Watson & Jamieson, 1995; Kaneko *et al,* 1996), having been performed successfully. Thoracoscopic oesophageal surgery (Bessell *et al,* 1994) and lung resections (Buhr *et al,* 1995) have also been performed. These laparoscopic techniques have been applied by surgeons to reduce the access related morbidity associated with conventional abdominal and thoracic incisions. The advantages are a shorter hospital stay, decreased post operative pain (McMahon *et al,* 1994b) and earlier return to normal activity (Watson *et al,* 1995a). The application of laparoscopic techniques has extended to the diagnosis, assessment and therapeutic resection of malignant disease (Babineau *et al,* 1994; Bemelman *et al,* 1995). Laparoscopic colectomy for colonic carcinoma has gained popularity and laparoscopic colonic resections are being performed in many centres around the world (Ota, 1995).

The first report of a port site recurrence was by Döbrönte *et al* in 1978. Since then a number of case reports have appeared in the surgical literature describing metastases arising in the wounds for the introduction of the laparoscopic instruments into the abdominal or thoracic cavity, following tumour resection (Nduka *et al,* 1994). Metastases can be defined by the terms invasion, dissemination, implantation and growth of tumour emboli (Scanlon & Murthy, 1991).

Whilst this problem has been highlighted especially following laparoscopic resection of colonic (Alexander *et al,* 1993; O'Rourke *et al,* 1993; Prasad *et al,* 1994) and gallbladder carcinoma (Clair *et al,* 1993; Wibbenmeyer *et al,* 1995; Cotlar *et al,* 1996), wound metastasis following laparoscopy for pancreatic carcinoma, during which the primary tumour was not manipulated, has also been reported by several authors (Siriwardena & Samarji, 1993; Watson, 1995). As a consequence, many surgeons have expressed concern about the applicability of laparoscopic techniques to the surgical treatment of malignancy (O'Rourke *et al,* 1993; Buhr *et al,* 1995; Wexner *et al,* 1995).

Whilst laparoscopy often reduces the short term morbidity of surgery, by reducing wound related pain (McMahon *et al,* 1993) and speeds post-operative recovery (Watson *et al,* 1995a) enabling patients to return to work earlier, all of these short term benefits are irrelevant if the technique increases the risk of tumour recurrence.

Whether the incidence of wound metastases is increased following laparoscopic tumour surgery is controversial (Savalgi & Rosin, 1996). This problem may be more likely after laparoscopic surgery due to the laparoscopic approach promoting spread, implantation and metastatic growth (Nduka *et al,* 1994). This should preclude the laparoscopic resection of malignant disease. However, it is also possible that tumour metastasis is less likely following laparoscopic surgery, as post-operative immune function appears to be less disturbed by the laparoscopic approach (Allendorf *et al,* 1996). In this scenario, reports of wound metastases could be anecdotal and not reflect the true outcome of laparoscopic resection, which may be advantageous to patients undergoing tumour excision. Metastases may also reflect the tumour stage at the time of laparoscopic excision and therefore have no deleterious effect on patient survival, thereby not precluding the application of laparoscopy to malignancy (Nieveen van Dijkum *et al,* 1996).

Controversy therefore exists about the increased incidence of wound metastases following the application of laparoscopic surgery. However, the growing number of

reports in the literature clearly indicates that this is an important problem and therefore warrants careful study to better understand this phenomenon (Nduka *et al,* 1994).

1.4. Wound Recurrence following Open Surgery

The incidence of wound metastases following open surgery for malignancy is difficult to determine. Wound involvement has been reported following open surgery as an infrequent event. There are reports of wound recurrence following open surgery for gastric carcinoma (Fortner, 1960), carcinoma of the cervix (Greenlee *et al,* 1981) and carcinoma of the oesophagus (Recht *et al,* 1989).

In one large series of patients which is often quoted, the abdominal wall recurrence rate was looked at following open colonic resections for large bowel cancer and recurrence was reported in 11 out of 1603 patients who underwent curative resection, which is an incidence of 0.6% (Hughes *et al,* 1983). Most patients in this series with incision site recurrence had advanced disease at the time of initial surgery. Thus wound involvement by tumour following resection for malignancy is rare and all of them were in advanced malignancies (Hughes *et al,* 1983).

There are reports of recurrence of cervical carcinoma in the incision following a staging procedure (Stenson *et al,* 1990) and a rare case of squamous cell carcinoma implanting in the episiotomy site is found in literature (Greenlee *et al,* 1981). The seeding of malignancy following diagnostic fine needle aspiration is well documented in the literature. The implantation of tumour to the skin or abdominal wall has been noted to occur with fine needle aspiration (FNA). There are case reports of tumour spread to puncture sites following needle aspiration of intra-abdominal cavity (Quaghebeur *et al,* 1991). Tumour seeding in the biopsy tracts after FNA have been recorded with a frequency of five in 5,000 biopsies (Lundstedt *et al,* 1991). Even if this is an over estimate, it gives only a 0.1% incidence. In a report by Pasieka & Thompson, 1992, a rare complication of peritoneal seeding was caused by FNA biopsy of a carcinoid tumour. Pasieka & Thompson, 1992, also reported an incidence of needle

tract seeding of 0.5%. There have also been reports of tumour metastases occurring at drain sites (Chapman *et al,* 1989).

Implantation of tumour following thoracic tumour resections is rare in the literature (Downey *et al,* 1996) and usually involves only mesothelioma. Similarly, chest wall implants after oesophagectomy are only reported rarely (Recht *et al,* 1989).

Tumour seeding to incision sites, needle tracts, drain sites and sites of injury have been documented in the literature (Verbeek *et al,* 1990; Lundstedt *et al,* 1991; Grabau *et al,* 1993). These reports are of intra-abdominal tumours originating from the gastro-intestinal (GI) tract or the pelvic organs, implanting into incisions. However, these were considered a rarity or anecdotal.

It is well documented that the peritoneal cavity of patients with intra-abdominal malignant disease contains free viable tumour cells (Fermor *et al,* 1986). It is well recognised that free malignant cells in the peritoneal cavity can migrate and implant on other organs, the well known example being the Krukenburg tumour (Ackerman, 1953). It has also been demonstrated that there is spillage of tumour cells, following surgery for malignant disease in the abdomen (Hansen *et al,* 1995).

There is evidence from experimental work that tumour cells preferentially implant in sites of tissue injury such as incisions (Murthy *et al,* 1989). The humoral response in wound healing provides a good environment for growth of tumour cells, by protecting the cells from the host defence system and providing factors which aid in their growth (Murthy *et al,* 1991). It has been postulated that the wound recurrences are due to direct implantation of cells, which are shed in the operative field (Hansen *et al,* 1995). There are no well documented studies that have looked at the problem of wound recurrence of tumour in the open surgery era. The most quoted series is a retrospective study by Hughes *et al,* 1983, which has documented an incidence of around 1% wound

metastases, when all the wounds were taken into consideration and about 0.68% when the recurrence in the laparotomy incision alone was taken into account.

It is possible that there may be under-reporting of this complication or it is not recognised. This is unlikely to be the case, however, considering such an incidence will have an adverse impact on the survival and quality of life of the patient. These instances would be imprinted in surgeons' memories, because wound recurrence of malignancy is always a difficult problem to treat. The standard textbooks in surgery do not consider this as a recognised complication of surgery for malignant disease (*Davis-Christopher Textbook of Surgery*; Rains & Ritchie, 1984; *Oxford Textbook of Surgery*). Hence, one may assume that this problem was not common during the era of open surgery to register caution in the minds of surgeons. Most surgeons would have seen only a few instances of wound metastases in their lifetime. Thus the spread of tumour to wounds is not a new phenomenon, but rare after open surgery.

1.5. Wound Metastases following Laparoscopic Surgery

This problem is not new, as the gynaecological literature also describes this phenomenon following laparoscopy for ovarian carcinoma (Hsiu *et al,* 1986). The earliest report was of a case of cutaneous metastasis appearing in a patient with ovarian carcinoma by Döbrönte in 1978. Metastases occurred in the port site wounds following laparoscopy of this patient with ovarian carcinoma and malignant ascites (Döbrönte *et al,* 1978). Since that time, there has been a steady flow of new case reports appearing in literature.

Unfortunately, whilst anecdotal reports may provide evidence of patients developing laparoscopic wound metastases, an overall incidence cannot be calculated without knowledge of the appropriate denominator i.e. the number of patients undergoing such laparoscopic procedures.

The true incidence and impact of this newly emerging problem is difficult to evaluate. Patients who are likely to develop this problem may do so in the next few years, as these techniques have only been widely practised recently. Thus the real importance of these metastases is difficult to assess. There is also an understandable and inevitable delay in communication through surgical literature. The reports so far may be only the "tip of the iceberg".

The recent surgical literature contains an increasing number of reports of wound metastases following laparoscopic procedures for intra-abdominal malignancy. Wound metastasis following laparoscopic manipulation has been reported in patients with gallbladder carcinoma (Jacobi *et al,* 1995; Horvath *et al,* 1996), ovarian carcinoma (Stockdale & Pocock, 1985; Gleeson *et al,* 1993), colorectal carcinoma (Kazemier *et al,* 1995; Jacquet & Sugarbaker, 1996), gastric carcinoma (Cava *et al,* 1990), pancreatic carcinoma (Siriwardena & Samarji, 1993), hepatocellular carcinoma (Russi *et al,* 1992)

and bladder cancer (Andersen & Steven, 1995). A review by Nduka *et al*, 1994, has highlighted a number of case reports describing this complication following the laparoscopic resection of colonic and gallbladder carcinoma.

It is arguable that the presence of a port site tumour, is an indication of the behaviour of the tumour or the intraperitoneal stage of spread of the tumour (Cook & Dehn, 1996). Although in many of the reported cases there was already advanced disease, port site recurrence has also occurred in the presence of tumours with low malignant potential. This has occurred in patients with early disease, such as carcinoma *in situ* in the gallbladder (Wibbenmeyer *et al,* 1995), borderline ovarian tumour (Shepherd *et al,* 1994) and Dukes A colorectal carcinoma (Prasad *et al,* 1994), and suggests that wound metastasis is not entirely dependent on tumour stage. It has occurred following laparoscopy in the presence of pancreatic carcinoma, even when tumour dissection or manipulation was not performed (Siriwardena & Samarji, 1993). This phenomenon is not unique to laparoscopy, but has also been seen in thoracoscopic resections of lung malignancies (Downey *et al,* 1996).

Most of the port site recurrences reported so far in the literature have occurred as early as 1 week and as late as 10 months (Nduka *et al,* 1994). In the review of the problem by Nduka *et al,* two cases are elaborated: one patient with non-invasive carcinoma of the gallbladder and the other who underwent a laparoscopic abdominal-perineal resection of a rectal carcinoma. The lymph nodes were involved, but the radial margins were free. Both of the patients developed port site metastases and had a poor outcome. These patients presented with nodules at the site where the port for the laparoscopic instrument was inserted during the initial surgery.

In the patient with carcinoma of the gallbladder, the time of recurrence following laparoscopic surgery was only four weeks and all the port sites were involved. For the group the median time to presentation from laparoscopy was 6 weeks. These lesions

had a typical hard and craggy feel with attachment to skin and pain was the predominant symptom (Nduka *et al,* 1994).

Although the impact of port site recurrence following surgery in malignant disease is not fully understood in respect to disease stage and survival, when this problem was encountered in open surgery it had an adverse bearing on the control of the disease and survival (Fortner, 1960). Judging by the outcome of the reported cases so far following laparoscopic surgery, it is logical to think that these recurrences will have an adverse impact on disease staging and survival, especially when patients with early disease develop this complication (Fry *et al,* 1995; Cotlar *et al,* 1996).

The increasing number of reports of wound metastasis following laparoscopic surgery for malignant disease is a disturbing and controversial phenomenon (Nduka *et al,* 1994; Savalgi & Rosin, 1996). The significance of this event is uncertain. Whilst many surgeons have been concerned that metastasis may adversely affect patient survival (Wexner *et al,* 1995; Volz *et al,* 1997), others have believed that the true incidence was unlikely to be greater than that seen following conventional open surgery, or that the problem only occurred following laparoscopy for advanced poor prognosis tumours (Cook & Dehn, 1996).

1.6. Laparoscopic Environment and Wound Metastases

There are many factors inherent in the performance of laparoscopic surgery and the environment created by it, that may enhance the risk of wound metastases:

a. There is a loss of tactile sensation by using the laparoscopic instruments and the operating manoeuvres are possibly less dextrous compared to conventional surgery, resulting in much rougher handling of tissues. This may produce greater spillage of tumour cells during laparoscopic procedures (Nduka *et al,* 1994). The opening up of tissue planes may be more extensive, creating a larger raw area. The opportunity to detect lesions that are more palpable and less visible may be lost (Siriwardena & Samarji, 1993).

b. There is a frequent change and repetitive movement of instruments during laparoscopic procedures leading to increased and repetitive contact between the abdominal wall and instruments. Malignant cells have been shown to collect in instruments (Gertsch *et al,* 1992). These cells could be deposited on the wounds.

c. The tissue removed during laparoscopy is often larger than the incisions for the introduction of ports, causing much shearing and handling of the tissue when removing it through these small openings. This may predispose to the direct implantation of tumour cells into these wounds (Alexander *et al,* 1993).

However, some of the reports of port site metastases indicate that there are other ill understood mechanisms which may play a part, as seen by the following reports:

1. Patients have developed wound metastases in the laparoscopic port site wounds and not the incision wounds made for open surgery, when procedures were converted to open surgery at the same time (Jacobi *et al,* 1995).

2. Patients developed port site metastases even when the lesions were not handled or visualised (Siriwardena & Samarji, 1993).

3. Metastases have occurred in port sites where there was no contact with the tumour or instruments handling the tumour (John *et al,* 1995; Rae *et al,* 1995).

4. Port site metastases have occurred following removal of very early carcinomas, where the chances of shedding malignant cells are minimal (Prasad *et al,* 1994).

5. This phenomenon does not seem to be unique to any particular tumour or procedure, there are reports of this occurring following laparoscopic surgery for malignant disease of the ovary, stomach, lymph nodes, pancreas, colon and gallbladder (Stenson *et al,* 1990; Keate & Shaffer, 1992; Aractingi *et al,* 1993; Walsh *et al,* 1993; Fry *et al,* 1995).

The possible mechanism for port site metastases may depend on factors that affect the general response of the patient, such as: metabolic and immunologic status, surgical trauma in the presence of malignant disease, or influences of the laparoscopic procedure and environment. There are many factors during the laparoscopic procedure which have been postulated to play a role in the development of this phenomenon, such as:

a. Direct transfer of tumour cells to wound sites by repetitive movement of instruments (Keate & Shaffer, 1992).

b. Gas, used to create the pneumoperitoneum, depositing tumour cells on the instruments and cannulae used for ports (Hewett *et al,* 1996).

c. The circulation of gas during maintenance of pneumoperitoneum may physically cause dispersion or redistribution of cells (Taffinder & Champault, 1996).

d. High pressure and high flow leaks of insufflating gas, around port sites and during repetitive movement of instruments, may predispose malignant cells by a "venturi" effect (Kazemier *et al,* 1995).

e. Circulating gas may alter the immune and metabolic response of the peritoneum, which may affect the way tumour cells implant and grow (Evrard *et al,* 1996).

f. Local factors in the wound healing and inflammatory response may be altered, to enhance tumour cell growth (Jacobi *et al,* 1995).

It is possible that the development of port site metastases is due to direct implantation. In some of the case reports there was no evidence of other peritoneal dissemination, apart from those to the port site wounds. Hence, a local implantation mechanism is feasible.

There is clinical and experimental evidence to indicate that surgical trauma facilitates tumour metastases (Agostino & Cliffton, 1963). Traumatised tissue has been shown to enhance growth of tumour cells. Experimental studies have shown that the frequency of implantation of malignant cells is enhanced, when these cells are presented to wounds during the early stage of healing. It has been demonstrated that tumour spreads preferentially to recently traumatised tissue (Murthy *et al,* 1989). Growth factors such as transforming growth factor- alpha (TGF–α) which are involved in the healing of wounds, may also promote proliferation of tumours. Trocar puncture sites and incisions are both rich in fibrin gel, fibronectin and platelets. Tumour cells may be entrapped in the fibrin gel, thereby creating a fertile substratum for tumour adhesion, protection against host defences and nutrition for tumour growth. The implanted cells may benefit from the alteration in host immunity or local factors released from the wound (Murthy *et al,* 1991).

It is well documented that free tumour cells are present in the peritoneal cavity in many malignant conditions (Iitsuka *et al,* 1979). It has also been demonstrated that viable tumour cells are liberated into the peritoneal cavity during resection for malignant disease (Symes *et al,* 1984). Implantation of exfoliated tumour cells has been demonstrated to occur. These cells may implant in the wound by direct contamination

from instruments as has been demonstrated in stapling instruments (Gertsch *et al,* 1992). The direct implantation mechanism does not explain the development of tumour in port sites where contamination is remote and in those through which the primary tumour was not handled. This also does not explain the occurrence of port site metastases in patients with *in situ* carcinoma (Wibbenmeyer *et al,* 1995) and where the tumour has not been manipulated (Siriwardena & Samarji, 1993; Watson, 1995).

It has been postulated that exfoliated tumour cells become air borne during pneumoperitoneum and are deposited or trapped on most intraperitoneal surfaces (Nduka *et al,* 1994). It has also been postulated that leakage of carbon dioxide around trocars or through trocar sites, might cause seeding of these sites with malignant cells carried by gas (Birkett, 1995). The so called "chimney effect" has also been postulated, where viable tumour cells are trapped at port sites, due to leaks around the port sites causing a local high gas flow (Bouvy *et al,* 1996b; Hubens *et al,* 1996). However, there is no evidence to date from clinical or experimental work to substantiate these theories.

Thus no single mechanism can explain the development of port site metastases. It is possible that the problem of port site metastases may be multifactorial.

1.7. Strategies to Prevent this Phenomenon.

As increasing attention to this problem arose only recently, there are no tested strategies to prevent the problem. The solutions should aim to reduce the amount of free malignant cells liberated into the peritoneum during or immediately after the procedure; or to prevent the malignant cells from coming into contact with the wounds or raw surfaces.

The use of intraperitoneal chemotherapy for gastric carcinoma is a proven modality of therapy and its use has been advocated before closure of wounds in laparotomy (Umpleby & Williamson, 1984). However the role of intraperitoneal chemotherapy in preventing port site recurrence has yet to be investigated.

Other potential solutions are cytotoxic washouts and wound protectors (Sandor *et al,* 1995; Jacquet & Sugarbaker, 1996). Sterilisation of the wound by CO_2 laser has also been suggested in the past (Lanzafame *et al,* 1988). Other potential solutions are slow desufflation and the performance of laparoscopic procedures without using gas i.e. gasless laparoscopy (Jones *et al,* 1995). The latter is an attractive option to be considered, as it may eliminate the influences of pneumoperitoneum.

1.8. Morbidity associated with Development of Port Site Recurrences

Whilst it is possible that the life expectancy of patients undergoing laparoscopic surgery for advanced malignancy may not be shortened by subsequent port site metastases, it is disturbing that some wound metastases have occurred in patients who had early stage carcinomas resected. It is logical to assume cutaneous seeding of cancer following laparoscopy will, in the case of a localised malignancy, hasten death rather than retard death, even in situations where the primary treatment is only palliative. This can be ascertained only by follow-up studies on patients who develop this problem. Port site metastases may be very distressing and difficult to treat and may significantly reduce the quality of life. There is little doubt that this is an important problem considering the ever growing number of cases being reported in literature.

Data from Other Experimental Studies

At the start of this work, the only information regarding port site metastasis was from case reports in the literature. The importance of this phenomenon is attested to by the considerable amount of information that has appeared since this study commenced. These studies and experiments are discussed in the Discussion section of this thesis (Chapter 14), in the context of the findings from this work.

CHAPTER 2 METHODS

2.1. Background and aims of this project

It is predicted that in the future, port site metastases will become an increasingly recognised problem following laparoscopic surgery (Nduka *et al,* 1994). Studies to understand the true magnitude of this problem and the mechanisms involved need to be undertaken with urgency. Until this problem is fully evaluated, the future role of laparoscopic surgery in malignancy will be uncertain.

The best way to determine the risk and significance of wound metastases is by conducting a prospective randomised trial of laparoscopic versus conventional surgery, in patients undergoing surgery for malignancy. However, this may require large numbers to achieve significance and results are unlikely to be forthcoming for many years. An alternative approach is to develop an animal model in which a malignant tumour can be grown successfully and consistently (Lundy *et al,* 1979). The animal should tolerate and recover from the operative procedure and live long enough to study the effect of surgical intervention on tumour metastases.

The aims of the body of work presented in this thesis are therefore:

1) To develop an animal model in which a malignant tumour could be grown reliably and in a location that would enable manipulation by open or laparoscopic surgery.

2) To develop the appropriate techniques and instrumentation for laparoscopy in an animal model, including minimising the effects of laparoscopy on the animal.

3) To study the effect of laparoscopy with CO_2 pneumoperitoneum on the implantation, dissemination and growth of malignant tumour.

4) To study the mechanisms involved in the spread of tumour in the presence of CO_2 pneumoperitoneum.

2.2. Animal Model and Tumour Characteristics

Animal models are used for research in different aspects of cancer, including metastatic spread and immune response. Rodents are preferred in large part because of the availability of inbred strains, which provide a consistent genetic basis for interpretation of results. The rat model is convenient because of it's large abdominal size, which makes it suitable for surgical interventions (Frey, 1997).

The Dark Agouti (DA) rat and the Dark Agouti mammary adenocarcinoma (DAMA) were chosen as the animal and tumour of choice to develop a model for the work described in this thesis, for the following reasons:

a. The DA rat is an immunocompetent species and so the observed experimental effects on this model could be extrapolated to the human situation.

b. The baseline physiological metabolic and haematological characteristics have been defined.

c. The Dark Agouti mammary adenocarcinoma is a tumour native to the species.

d. The tumour has been found to grow reliably and consistently after inoculation and tissue transplantation (Rofe *et al,* 1986; Rofe *et al,* 1989; Coyle *et al,* 1990; Rofe *et al,* 1994).

Animal Supply

Dark Agouti (DA) rats are an inbred immunocompetent species of rats. The animals used in all the studies were male DA rats, except the carrier rats which were females. Inbred male and female DA rats were obtained from the Institute of Medical and Veterinary Science (IMVS) animal breeding facility at Gilles Plains, South Australia. The genetic profile of the inbred strain is checked yearly by the supplier. The rats weighed 210 - 250g.

Animal maintenance

The rats were housed in perspex cages in an animal house maintained at 22 ± 1°C with a 14h light/ 10h dark cycle. Rats were fed mouse M & V cubes (Milling Industries, Adelaide). The rats were caged in groups of two, while food and water were available *ad libitum* throughout the study period. The animals were studied in the fed state.

Tumour source

The tumour used was a Dark Agouti mammary adenocarcinoma (DAMA), originally obtained from the Department of Pathology and Immunology, Monash University Medical School, Victoria, Australia. This tumour has been maintained in Adelaide and used in the Institute of Medical and Veterinary Science (IMVS) Department of Clinical Biochemistry, for the study of the metabolic effects of tumours. The tumour arose spontaneously in female DA rats in 1972 and has been maintained by transplanting from animal to animal. Hereafter the tumour is referred to as DAMA.

Maintenance of tumour

The DAMA was passed in syngeneic rats by injecting a sterile suspension of tumour cells or by transplanting small pieces of tissue. The former is the preferred method of transplantation as this markedly decreases the risk of infection. The following method was used to produce a cell suspension for transplantation (Nilsson *et al,* 1996).

The DAMA has been maintained by injecting tumour cell suspension into the flanks of recipient rats by the following technique:

Viable DAMA cells were prepared by homogenising fresh tumours propagated in, and resected from, female carrier rats. Subcutaneous tumours of approximately 5g were excised and dissected free from necrotic and connective tissue.

The tumour was diced and washed in phosphate buffered saline (PBS Sterile 10 mmol/ L sodium phosphate buffer pH 7.0, containing 0.15 mol/ L sodium chloride). Tissue was homogenised in a motor driven Potter Elvehjen homogeniser (radial clearance 0.5 mm) and the crude debris removed by filtration through sterile gauze. The cell suspension was centrifuged three times in 10 volumes of PBS at 400 g each for 1 minute. The viability of cell suspensions was assessed by trypan blue (Hopkins & Williams, Essex, England CI 23850) exclusion (trypan blue in 0.5% saline) and the cell number determined using a Neubauer counting chamber (Improved Neubauer, Weber, England) (depth 0.1mm, 1/400 m^2). The final concentration was adjusted to give 2 x 10^7 cells in 0.2 ml of sterile PBS. Cells were injected into the abdominal wall of each rat under light halothane anaesthesia. After a lag period of 7 - 10 days, the tumour becomes palpable and grows rapidly at approximately 1 gram/ day.

This tumour could also be transplanted by direct transplantation of tumour tissue. These tumour cells have also been successfully grown in tissue culture media and tumours could be produced by injecting rats with cells grown in culture.

The tumour contained nests of large, polymorphic cells surrounded by a vascular stroma. Neoplastic cells were surrounded by abundant, eosinophilic cytoplasm and exhibited a lack of glandular differentiation. The nuclei were large, vesicular, contained prominent nucleoli and demonstrated a mitotic index of 3.95 ± 1.35 cells per high power field (x100). The ultra structure of DAMA tissue is consistent with that of adenocarcinoma of the mammary gland. The cells have endoplasmic reticulum which is dense and granular; irregularly shaped nuclei and prominent intra-cytoplasmic vacuoles, which are lined with microvilli. The size and distribution of these vacuoles vary greatly from cell to cell (Bourgeois, 1992).

Cytogenetic studies performed on cultured DAMA cells show that the cell line is characterised by near tetraploidy (83- 85 chromosomes). Closer examination of the

chromosomes revealed trisomy of pair 2 chromosomes and a metacentric marker formed by centric fusion of two pair of 3 chromosomes. The findings are constant features of karyotype. The DNA concentration of DAMA cells is 22.9 ± 0.8 pg/ cell and the total protein concentration is 0.5 ± 0.01 ng/ cell (Bourgeois, 1992).

The tumour associated vasculature consisted of irregular blood vessels, forming extensive networks. The vessels were composed only of a thin layer of endothelium and were distributed irregularly throughout the tumour mass. Vascular tissue was just observable in tumours 4 days after implantation. The relative vascular volume then increased exponentially to reach a plateau of approximately 6% at 12 days after implantation. As the size of the tumour increased, the diameter of some blood vessels also increased (Nilsson *et al,* 1996).

Immune composition of DAMA

Immunohistochemical characterisation of immune cell infiltrates of the DAMA showed that the macrophages (ca 140) and CD4 (ca 130) positive cells were the most prominent infiltrates. B cell staining was absent in the primary tumours (*personal communication* Zhang Xu Dong, 1997).

Natural history of tumour growth

The injection of the tumour cell suspension into the abdominal wall produced a solid neoplasm, which became palpable 6 to 8 days after transplantation. Growth was exponential with tumour size increasing 12 days after implantation. At 12 days post implantation, the tumour represents 5 - 10 % of body weight. However, the lag period from the time of inoculation to when the tumour is palpable can be decreased by increasing the number of tumour cells inoculated. Previous investigators have reported that there is a lack of metastases with this tumour after numerous autopsies on tumour bearing rats. Work described in this thesis also confirms this finding, except in animals where the tumour was surgically lacerated. The autopsies of rats in the studies

described in this thesis occurred when the tumours had grown to 15 - 20 % of body weight (Nilsson *et al,* 1996).

Establishment of the model for the present study

After a pilot study it was found that a tumour of the size of 20 - 25 mm in diameter could be grown consistently on the anterior abdominal wall of the DA rat. This was achieved by injecting a suspension of 200 µl of DAMA cells prepared in the method described earlier in this chapter. At day 7 after inoculation this tumour could be palpated.

This DAMA model has several characteristics in common with the human counterpart. In particular DAMA displays histological features typical of an adenocarcinoma, being characterised by a lack of glandular differentiation, a high mitotic index, tetraploidy and a high rate of glycolysis. A lack of metastases in the host also ensures that single lesion studies and animal responses are not confounded by secondary tumour deposits. Thus DAMA in the DA rat is an appropriate model for studying the histological and metabolic responses to the laparoscopic environment and the effect of this environment on tumour growth, dissemination and implantation. It is recognised that the findings from these studies may not be applicable to all tumours. Nevertheless, as will be demonstrated, this model does provide an insight into the role of the various components of laparoscopic environment on the dissemination of tumour cells.

Ethical approval

The protocols for all the animal studies reported in this thesis were approved by the Animal Ethics Committees of the Institute of Medical and Veterinary Science (Appl No 48/95) and The University of Adelaide, South Australia (Appl No 095/95).

2.3. Operative Techniques

All procedures were performed under general anaesthesia. The anaesthesia was induced and maintained by a combination of oxygen, nitrous oxide and halothane. The rats were initially anaesthetised in a perspex box into which nitrous oxide, oxygen and halothane were circulated. Once the rats were asleep, anaesthesia was administered to the animal using a custom made tight fitting mask, into which the snout of the rat was introduced. The anaesthesia was maintained with halothane (Fluothane, Zeneca Ltd, UK) in combination with oxygen and nitrous oxide. The delivery of gases and halothane was titrated and controlled by using a direct circuit to a CIG Midget-III anaesthetic machine, incorporating a Cryopane Fluothane Vapouriser. The technique of surgical procedures used during all the studies were standardised as follows:

Laparotomy

All the animals undergoing laparotomy or open procedure had a midline abdominal incision of 3 cm length. This incision was adequate in exposing the abdominal cavity and its contents. This exposure also provided adequate access to perform any manipulation in the peritoneal cavity or on any of the intra abdominal organs. The abdomen was kept open during the time of the procedure and the peritoneal cavity was left exposed. After completion of the procedure the abdomen was closed with 3-0 Prolene in two layers.

Laparoscopy

Pneumoperitoneum was achieved using a conventional Veress needle (Surgineedle - 18 gauge i.e. 18G, 150 mm long, Autosuture), introduced through a stab wound made on the right side of the midline. The Veress needle was introduced through a disposable mini-cannula (MicroLap Introducer Model LT 1500- MF-Imagyn Medical, Laguna Niguel, California, USA). The mini-cannula was left *in situ* after the establishment of the pneumoperitoneum and the Veress needle was removed.

The mini-cannula was provided with a side channel, which was connected through a 0.5 mm diameter tubing to an insufflator (Karl Storz, model 26012B, Germany). This provided a constant flow of gas to maintain the pneumoperitoneum.

The mini-cannula was used to provide access for a 2 mm diameter mini-laparoscope (Imagyn Medical, California, USA) with a focal length of 1 cm and a length of 275 mm. This mini laparoscope was attached to a conventional colour CCD laparoscopy camera (Panasonic WV= KS152). The light was provided by a cold light fountain (450V-Karl Storz, Germany). The whole system was connected to a video-monitor (Panasonic, Japan).

Two additional "ports" were inserted: an 18 gauge cannula in the left hypochondrium, which was left open throughout the procedure to vent the insufflation gas; and a 16 gauge cannula in the left lower quadrant, which was used to provide access for a needle used for tumour laceration. At the end of the procedure the puncture sites used for the cannulae were closed with sutures.

Gasless laparoscopy

A Veress needle was introduced into the peritoneal cavity using the same technique described earlier, with its intraperitoneal position ascertained by the combination of: injection of a small volume of air using a syringe and direct visualisation with the laparoscope. The abdominal wall was then suspended from a wire frame, by passing multiple sutures through the overlying skin and anchoring these to the frame. The passive inflow of room air into the peritoneal cavity provided an adequate laparoscopic working space. As with the other group, a large intravenous cannula was then inserted opposite the tumour, to provide access for instrumentation. The sutures used for suspending the abdominal wall were removed at the end of the procedure and the puncture sites were closed with 3-0 Prolene sutures.

Peri operative monitoring

The respiratory and general status of the animals was monitored and care was taken to ensure that there was no distress or respiratory embarrassment.

Post operative monitoring

The animals were monitored every day, to ensure that their food and water intake was adequate. The animals were also carefully monitored for signs of discomfort or distress. The DA rats tolerated laparotomy and laparoscopy well. They rapidly recovered from the effect of anaesthesia and surgery. At day 7 following inoculation, the tumours were readily visible at either laparotomy or laparoscopy and were still covered by a thin layer of peritoneum.

2.4. Autopsy Examination and Histopathology

All animals were killed by cervical dislocation under general anaesthesia, or by intra-cardiac injection of potassium chloride (KCl) under anaesthesia. Seven days after the operative procedure the animals were killed, the abdominal cavity opened and the dimensions of the primary tumour measured. Both the laparotomy and laparoscopy access wounds were examined directly for evidence of macroscopic tumour metastasis, before excising the wounds for histopathological examination. Any other areas suspicious of tumour involvement or metastasis were also excised for histological examination.

Tumour size

The dimensions of the primary tumour were measured from the outer aspect of the abdominal wall.

Histopathological processing and examination of port site wounds and peritoneal surface

Excised specimens were fixed and stored in buffered formalin. The fur was removed from the specimen and the tissue was cut to 3 mm width pieces and processed in tissue cassettes. After the tissue pieces were embedded in paraffin blocks, they were cut with a microtome to a thickness of 5μm and placed on glass slides. The slides were dried overnight at 37°C and then dewaxed.

Haematoxylin and eosin staining was performed by initially placing the slides in two washes of Histoclear or xylene for 2 minutes each; then re-hydrated by dipping in 3 baths of alcohol. The slides were rinsed in deionised water and PBS before being placed in haematoxylin for 3 minutes. The excess stain was removed by rinsing the slides in water and then acetyl alcohol and the remaining stain in the tissue was fixed in lithium carbonate for one minute. The counter staining with eosin was performed for 1

minute. Excess of this stain was removed by rinsing, after which the slides were soaked in PBS for 30 seconds.

The slides were then placed in 3 alcohol baths and 3 Histoclear baths for a minute each. A coverslip was then placed on the stained tissue section using Dipex mounting medium and allowed to dry overnight.

All specimens were examined by a histopathologist, who was blind to the type of procedure undertaken and the anatomical site of origin of the specimens.

CHAPTER 3 SOLID TUMOUR MODEL

3.1. Comparison of the Spread of Tumour following Laceration of the Abdominal Wall Malignancy during Open and Laparoscopic Surgery.

Aims of the Study

The aim of this study was to determine whether the application of laparoscopic techniques to the manipulation of malignant abdominal tumour implanted in DA rat model leads to an increased risk of tumour dissemination and implantation within the peritoneal cavity and abdominal wall wounds.

Materials and Methods

Previous experience using the DAMA tumour cell line in DA rat has shown that a malignant tumour can be reliably implanted in laboratory rats by the injection of a cell suspension, providing a model that allows for the investigation of the effect of laparoscopy and laparoscopic manipulation on intra-abdominal malignancy and the pattern of tumour implantation.

Forty-two male Dark Agouti (DA) rats weighing 240 to 280 g (median 250g) were injected in the left anterior abdominal musculature, with a suspension of 200 µl (i.e. 2 x 10^7 cells) of mammary adenocarcinoma (DAMA) tumour cells. Tumours implanted in the lateral abdominal wall were still covered by a thin layer of peritoneum at this stage and were readily visible at either laparoscopic or open surgery.

Seven days after tumour implantation the rats underwent an operative procedure in one of the following groups (Figure 3.1):

1) **CONTROL GROUP**

anaesthesia only	6 rats

2) **LAPAROTOMY**

laparotomy with no manipulation of tumour	6 rats
laparotomy with laceration of tumour	12 rats

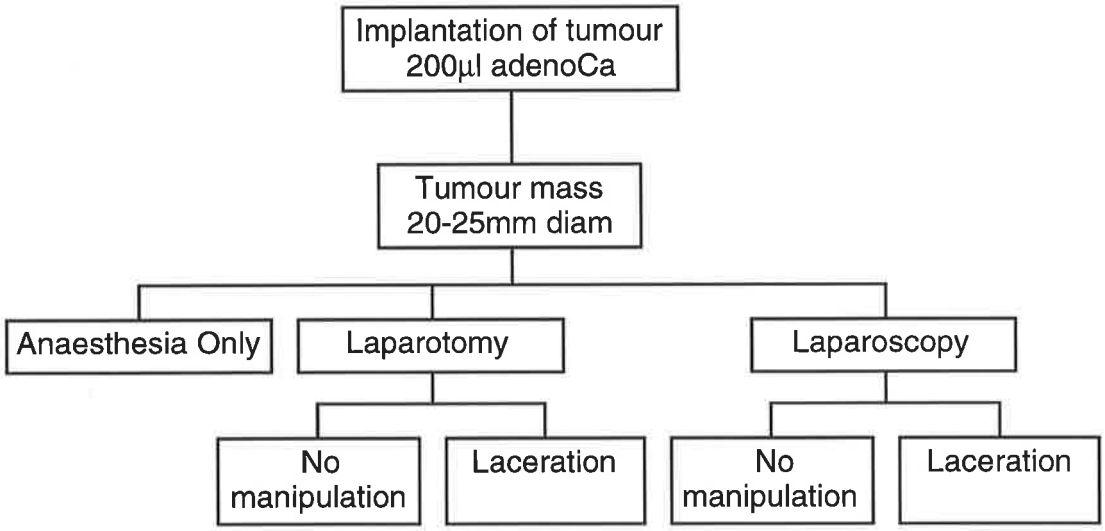
3) **LAPAROSCOPY**

laparoscopy with no manipulation of tumour	6 rats
laparoscopy with laceration of tumour	12 rats

All rats were anaesthetised during these procedures and all surgical procedures were performed under sterile operating conditions.

FIGURE 3.1

SCHEME OF THE SOLID TUMOUR STUDY



Control Group

Rats in the control group were anaesthetised for a 30 minute period, but underwent no operative procedure.

Laparotomy Group

Rats undergoing laparotomy were operated through a midline incision of 3 cm length, exposing the abdominal cavity and its contents. Tumour was visibly bulging into the abdominal cavity, but was covered by peritoneum. In the tumour laceration group, the tumour capsule was lacerated once through the covering peritoneum using the tip of an 18 gauge needle. The abdomen was subsequently closed with 3-0 Prolene sutures in 2 layers.

Laparoscopy Group

In rats undergoing laparoscopy, pneumoperitoneum was achieved using a conventional Veress needle placed through an umbilical stab wound. A disposable mini-cannula was then slid over the needle to provide access for a 2 mm mini-laparoscope, which enabled continuous insufflation of gas. Carbon dioxide was insufflated at a rate of 0.1 L/ min at a pressure of 2 mmHg. The previously implanted tumour was easily seen to be bulging into the abdominal cavity when viewed through the laparoscope. An 18 gauge intravenous cannula was introduced through the abdominal wall opposite the tumour, providing access for subsequent instrumentation. In the laceration group, a long needle was then introduced through this port, to facilitate a single tumour laceration under direct vision. Following tumour laceration, the cannula was left *in situ* as a laparoscopic 'cannula' and was used to vent insufflated gas. Pneumoperitoneum was maintained for 60 minutes with a constant gas flow through the venting cannula.

The non-laceration laparoscopy group underwent the same manoeuvres, with the exception of actual tumour laceration. Care was taken to ensure that the respiratory status of the animal was not compromised. After 60 minutes the insufflating and

venting cannula were removed and the puncture sites were closed with interrupted 3-0 Prolene sutures. All surgery was performed by the same investigator.

Autopsy and Histological examination

Seven days after the operative procedure the rats underwent autopsy, the tumour size measured, the peritoneum examined for tumour spread and tissue samples taken for subsequent histopathological examinations as described in the Chapter 2.

The weights of animals in each group were similar preoperatively and were not significantly altered by either tumour laceration or the surgical access technique.

Statistical Analysis

Statistical analysis of data sets was performed using the Mann-Whitney U test.

Results

Primary Tumour Growth

Primary implanted tumours in all groups (control, laparotomy and laparoscopy) enlarged in the week following general anaesthesia and associated surgery. Tumour growth is summarised in Table 3.1. The percentage of enlargement of the primary tumour following laparotomy was greater than that following laparoscopy (P = 0.04), or that seen in the control group (P = 0.09). Intra-operatively, the implanted tumours in the rats undergoing laparotomy were approximately 25% smaller in maximum dimension than those measured in either the control (P = 0.01) or laparoscopy groups (P = 0.05). Tumour size and growth were not related to the body weight.

TABLE 3.1

TUMOUR GROWTH FOLLOWING SURGERY

(maximum dimension in mm - median & range)

	Control	Laparoscopy	Laparotomy
	n = 6	n = 18	n = 18
Pre - op Size	33 (25-40)	30 (15-40)	23 (15-35)
7 Days Post- op Size	55 (45-70)	55 (40-70)	53 (30-70)
% Size Increase	78 (43-200)	93 (50-225)	133 (50-225)

Wound Metastasis

No macroscopic or microscopic tumour metastases developed in the wounds of rats undergoing either laparoscopy or laparotomy without tumour laceration. Tumour metastases were present in the wounds of rats undergoing tumour laceration by either a laparoscopic or open surgical technique (Table 3.2). Metastases were significantly more common in the laparoscopic group (P = 0.003).

TABLE 3.2

WOUND METASTASES ONE WEEK AFTER TUMOUR LACERATION

(number metastases/ total number rats)

	Laparoscopy	Laparotomy	P value
Macroscopic metastases	5/ 12	1/ 12	P = 0.15
Microscopic metastases	10/ 12	2/ 12	P = 0.003

The distribution of tumour metastases found in the port wounds is summarised in Table 3.3.

TABLE 3.3

LOCATION OF WOUND METASTASES FOLLOWING LAPAROSCOPIC TUMOUR LACERATION

LOCATION OF METASTASES	NUMBER
No tumour in either wound	2
Wound for laparoscope only	4
Wound for manipulating/ lacerating instrument only	4
Both wounds	2

Tumour Metastasis Within The Peritoneal Cavity

No tumour dissemination or peritoneal invasion was evident in the control, laparotomy or laparoscopy groups of rats that did not undergo tumour laceration. In the two groups that underwent tumour laceration there was no macroscopic evidence of tumour dissemination beyond the immediate site of tumour laceration. At the site of tumour laceration, tumour was seen to be growing through the peritoneal covering in all rats.

Discussion

This study describes a very high rate of wound metastases in rats undergoing laparoscopic tumour laceration, compared to rats undergoing an identical procedure at open surgery. There was a five-fold increase in the presence of tumour in wounds of animals undergoing laparoscopy and laceration of tumour. The tumour was present in the port wounds used for passage of the laparoscope, as well as that used for tumour laceration. This pattern of distribution of tumour cells suggests that direct implantation of tumour from a tumour laden instrument or specimen extraction, may not be the only mechanism involved in the development of port site metastases. It is of concern from the reports of port site metastases that tumour recurrence has occurred in port wounds through which no tumour or specimen was extracted and hence had no direct contact with the resected tumour (Siriwardena & Samarji, 1993; Jorgensen *et al,* 1995; Watson, 1995).

The possibility of direct wound contamination during specimen delivery, as well as several other aetiological theories, such as: the abnormal circulation of free intra-peritoneal tumour cells, due to the gas flow during pneumoperitoneum; or a chimney effect, due to gas leaking around laparoscopic ports with subsequent lodging of tumour cells in wounds, have been proposed (Nduka *et al,* 1994). It is possible that free cells lodge in laparoscopic wounds, implant and proliferate, due to some factor inherent in the laparoscopic procedure or environment. Tumour cells can preferentially grow in recently traumatised tissue (Murthy *et al,* 1989). In this model there were no metastases to other organs in the abdomen, such as the liver. There were no peritoneal implants, other than at the trocar sites or incision, in this study.

Other researchers have demonstrated reduced primary tumour growth following laparoscopy in animal models (Allendorf *et al,* 1995a). This has been postulated to be due to the beneficial immunological effects of laparoscopic surgery compared to open surgery (Allendorf *et al,* 1996). In this study a similar difference in the growth of primary tumour was observed. The differential growth of the primary tumours in this

study supports the observations of other studies in respect to tumour growth (Bouvy *et al,* 1997). However, the increased incidence of wound metastases suggests that this benefit may be annulled by the increased spread of tumour to wounds.

In the clinical situation the decreased growth of the primary tumour may have no practical significance in patients operated on laparoscopically for malignant disease, in whom malignant tissue is breached. In this study, no metastases developed in animals in which the tumour was not lacerated. This cannot be extrapolated to humans and it should not be assumed that laparoscopic tumour surgery is safe, if no macroscopic breach of the tumour capsule occurs during surgery.

The technical difficulties of laparoscopic dissection may make inadvertent tumour laceration more likely than during open surgery, with potentially more serious consequences for patients. It has also been demonstrated that free malignant cells are present in the peritoneal cavity even before the tumour is handled.

The results of this study add weight to the argument that there is an increased likelihood of wound metastases if malignant tumours are cut or lacerated during laparoscopic removal. This finding has an important implication to clinical situations, where laparoscopic surgery is performed for malignancy. This is a problem that must be addressed urgently.

This model offers a unique opportunity to investigate this issue further. This model can be used to manipulate the laparoscopic environment, in ways that may reduce or eliminate the likelihood of wound metastases.

CHAPTER 4 GAS AND GASLESS LAPAROSCOPY

4.1 The Difference in Spread of Abdominal Malignancy during Laparoscopy, with and without CO_2 Pneumoperitoneum.

Aims of the Study

To determine whether the likelihood of adverse effects on tumour spread caused by laparoscopic surgery can be reduced by the eliminating the use of pressurised insufflation of carbon dioxide. This study was conducted to investigate the incidence of port site metastases following tumour manipulation during 'conventional' and 'gasless' laparoscopic surgery.

Materials and Methods

The animal model and the tumour cells were prepared as described earlier (Chapter 2.2). For this study 24 DA rats weighing from 240 to 280 g had intra-abdominal tumours induced. After 7 days, tumours grew to approximately 1 to 2% of the body weight. All rats underwent an operative laparoscopic procedure under anaesthesia and sterile operating conditions. The respiratory status of the animals was closely monitored during the operation.

Laparoscopy with CO_2 insufflation

Twelve rats underwent laparoscopy with conventional CO_2 pneumoperitoneum. Pneumoperitoneum was established using a standardised protocol described in chapter 2. Carbon dioxide gas was then insufflated at a rate of 0.1 L/ min and at pressure of 2 mmHg, to provide effective laparoscopic exposure of the previously implanted tumour. A large intravenous cannula was then introduced through the abdominal wall opposite the tumour, providing access for subsequent instrumentation and venting of insufflated gas. The tumour capsule was lacerated in a standardised fashion under direct vision

through the access port. Following this, both cannulae were left *in situ* and insufflated gas was vented for a period of 60 minutes whilst a constant gas flow was maintained. Subsequently, all cannulae were removed and the abdominal wounds were closed with 3-0 Prolene sutures.

Laparoscopy without CO_2 insufflation (Gasless Technique)

A further 12 DA rats underwent surgery using an identical laparoscopic surgical protocol, but using a 'gasless' technique, which eliminated the need for CO_2 insufflation. A Veress needle was introduced into the peritoneal cavity using the same technique described earlier, with the intraperitoneal position ascertained by the combination of injection of a small volume of air using a syringe and direct visualisation with the laparoscope. The abdominal wall was then suspended from a wire frame, by passing multiple sutures through the overlying skin and anchoring them to the frame. The passive inflow of room air into the peritoneal cavity provided an adequate laparoscopic working space. As with the other group, a large intravenous cannula was then inserted opposite the tumour, to provide access for instrumentation. A long needle was introduced through this 'port' to achieve tumour laceration under direct vision. All ports were then left *in situ* for 60 minutes, before removal and the puncture sites were closed with 3-0 Prolene sutures.

Autopsy and Histopathology

Seven days after the operative procedure, all animals were killed, the abdominal cavity was opened and the dimensions of the primary tumour were measured. The peritoneum was examined for tumour spread. All wound sites were excised for histopathological examination along with any other areas suspected of having tumour involvement or metastasis. Histopathological techniques were applied as described in the methodology (Section 2.4 of Chapter 2).

Statistical analysis

Fisher's exact test was used for the analysis of data represented by contingency tables and the Mann Whitney U-test for the analysis of unpaired non-parametric data sets.

Results

All implanted primary tumours enlarged in the week following surgery, with tumour growth rates comparable between the two study groups. The primary tumour increased in size by a mean 98 % (range 29 to 200 %) in the conventional laparoscopy group, compared to 119 % (range 67 to 206 %) in the gasless group (P = 0.26, Mann-Whitney U-test).

TABLE 4.1

TUMOUR GROWTH FOLLOWING SURGERY

(maximum dimension in mm - median & range)

Laparoscopy	with CO_2	without CO_2	P value
	n = 12	n = 12	
% Size Increase	98 % (29 - 200 %)	119 % (67 - 201 %)	0.26

TABLE 4.2

WOUND METASTASES ONE WEEK AFTER TUMOUR LACERATION

(number metastases/ total number rats)

	Conventional Laparoscopy	Gasless Laparoscopy	P value
Macroscopic metastases	5/ 12	2/ 12	P = 0.37
Microscopic metastases	10/ 12	3/ 12	P = 0.012

Tumour metastases were found significantly more often in the wounds of rats undergoing laparoscopic tumour laceration during carbon dioxide insufflation, than following comparable gasless surgery (P = 0.012, Fisher's exact test, Table 4.2).

The distribution of tumour metastases found in the port wounds is summarised in Table 4.3, indicating no predilection for a particular trocar site in either group. No tumour dissemination or peritoneal invasion was evident beyond the site of primary tumour laceration and the abdominal wounds in any of the 24 experimental animals. At the site of tumour laceration, tumour was seen to be growing through the peritoneal covering in all instances.

TABLE 4.3

LOCATION OF WOUND METASTASES FOLLOWING TUMOUR LACERATION

Location of Metastases	Conventional Laparoscopy	Gasless Laparoscopy
No tumour in either wound	2	9
Wound for laparoscope only	4	1
Wound for lacerating instrument only	4	2
Both wounds	2	0

Discussion

In this study the growth of primary tumour following laparoscopy with and without gas was similar. The findings from this study clearly show that the incidence of wound metastasis was significantly reduced by eliminating the use of carbon dioxide insufflation under pressure. This reduction in the incidence of wound metastases was comparable to the rate seen following the equivalent open surgical procedure reported in the earlier study. In the present study, there were 3 metastases in 12 rats in the gasless laparoscopy group and in the laparotomy group from the earlier study there were 2 metastases in 12 rats. The results of this study and studies conducted by others, suggests that the use of carbon dioxide as insufflation gas is associated with the movement of cells to laparoscopic trocar wounds (Jones *et al,* 1995; Hewett *et al,* 1996; Thomas *et al,* 1996). These cells then implant and grow. These cells were liberated into the peritoneal cavity by the intentional breaching of the tumour capsule during laparoscopic manipulation during this study.

However, in the clinical situation, cells may either be free in the peritoneal cavity of humans undergoing surgery for malignancy (Tanida *et al,* 1982) or be liberated into the peritoneal cavity by breaching the tumour capsule (Zirngibl *et al,* 1990) during surgical manipulation. The presence of circulating gas seems to enhance the incidence of tumour spread to wounds (Jones *et al,* 1995). The removal of the insufflation gas eliminates one of the essential environmental components necessary for laparoscopic wound metastasis. Gasless laparoscopy has been advocated (Maher, 1995) to avoid the physiological consequences of a pneumoperitoneum, especially in patients with severe respiratory and cardiology co-morbidity (Ishizaki *et al,* 1993; Baraka *et al,* 1994; Chiu *et al,* 1995; McDermott *et al,* 1995). This is now feasible in clinical practice, as laparoscopic abdominal wall lifting devices are becoming commercially available (Smith *et al,* 1993). Further clinical studies will be necessary to investigate the efficacy of this technique and confirm the findings of the beneficial effect of gasless laparoscopy in patients with malignant disease, with respect to reduced spread of tumour to wounds.

The findings from this study, that elimination of carbon dioxide insufflation can reduce the incidence of wound metastases, is of major clinical importance. Further this experimental model is an appropriate means for devising studies and testing other such factors of the multifactorial mechanisms behind laparoscopic wound metastasis.

CHAPTER 5 FREE TUMOUR CELL MODEL

5.1. Free Tumour Cell Model

Background

In the past animal models to study the behaviour of tumour growth have used both implanted neoplasms as well as free cell suspensions (Lundy *et al,* 1979; Weese *et al,* 1986; Fujita *et al,* 1992; Frey, 1997). Free cell suspensions are more quantifiable in that the tumour inoculum used is measurable in terms of the number of cells introduced. Thus it is a definable stimulus of a tumour. Free tumour cell lines from many tumours have been found to grow reliably when introduced into the peritoneal cavity by direct instillation or by injection. These models of intraperitoneal induction of tumour have been used to assess the effect of therapeutic interventions, such as chemo-therapeutic agents or immune modulation (Frey, 1997). The DAMA tumour implanted onto the abdominal wall has not been found to metastasise or spread to the peritoneal cavity spontaneously. In this study, the feasibility and reliability of intraperitoneal growth of tumour following injection of free cells was assessed.

Aim of the study

To develop an alternative model to study the effects of various types of surgical procedures on the intraperitoneal pattern of implantation and growth of tumour. The introduction of a free tumour cell suspension into the peritoneal cavity instead of laceration of a pre-existing tumour, would remove the potential variability in the number of tumour cells liberated using the laceration technique.

Methods

Preparation of free cell suspension

Subcutaneous tumours of approximately 5 g were excised from the carriers and dissected from any surrounding tissue. The tumour was then diced and washed in

phosphate buffered saline (PBS, sterile 10 mmol/ L sodium phosphate buffer pH 7.0, containing 0.15 mol/ L sodium chloride). The tissue was homogenised in a motor driven Potter Elvehjen homogeniser (radial clearance 0.5 mm) and the crude debris removed by filtration through sterile gauze. The cell suspension was next centrifuged three times in 10 volumes of PBS at 400g, each for 1 minute.

The viability of the suspension was assessed by trypan blue (Hopkins & Williams, Essex, England, C.I. 23850) exclusion (trypan blue in 0.5% saline) and the cell number determined using a Neubauer counting chamber (Improved Neubauer, Weber, England, depth 0.1 mm, 1/400m^2). The final concentration was adjusted to give 2 x 10^7 cells in 200 µl of sterile PBS. This was injected into left upper quadrant of the peritoneal cavity of animals under anaesthesia.

Intraperitoneal injection and optimal dose

All intraperitoneal (IP) injections were performed under anaesthesia. The cells were introduced through a standard site on the abdominal wall. The cells were injected into the left upper quadrant, just below the costal margin. The tumour cell dose was determined in a pilot study where tumour implantation and growth patterns following the introduction of varying dilutions of the cell suspension were investigated. When more concentrated suspensions (2 to 4 x 10^7 cells) were used, tumour spread was too prolific and difficult to quantify. When weaker dilutions were used (2 x 10^6 cells in 200 µl of sterile PBS), tumour growth was sparse.

The animals were housed in the laboratory for six days. Food and water consumption was normal within 24 hours of the surgical procedures.

Assessment of tumour implantation and growth

Six days after tumour implantation all animals were killed and an autopsy was performed. All animals that underwent IP injection of tumour cells developed a tumour

within the peritoneal cavity. The abdomen was examined for tumour deposits and the tumour implantation pattern assessed using a standardised scoring system.

Peritoneal Cancer Index

The abdominal cavity was divided into six sectors and the presence and pattern of tumour implantation documented for each sector. The density of tumour implantation was scored according to the peritoneal cancer index proposed by Eggermont:

0 = no intraperitoneal tumour

1 = less than three minute tumour foci

2 = moderate tumour

3 = abundant or confluent tumour (Eggermont *et al,* 1988).

Each sector was therefore scored for the presence or absence of tumour deposits and for tumour density. Representative samples were examined histologically to confirm the macroscopic assessment. All surgical access wounds were examined for macroscopic evidence of tumour implantation.

5.2. Free Tumour Cell Study:

The Effect of CO_2 Pneumoperitoneum on the Implantation and Growth of Tumour following Intraperitoneal Injection of Free Tumour Cells

Introduction

Laparoscopic techniques have now been applied widely by surgeons in an attempt to reduce the access related morbidity associated with conventional abdominal and thoracic incisions (Clayman *et al,* 1991; Becker *et al,* 1993; Charnley, 1994; Cuschieri *et al,* 1996). A logical progression has been the application of laparoscopy to the diagnosis, assessment and therapeutic resection of malignant disease (Boutin & Rey, 1993; Cole *et al,* 1994; Bemelman *et al,* 1995; Bogen *et al,* 1996; Conlon *et al,* 1996). However, because of an increasing number of published reports describing metastases to port wounds following laparoscopic surgery for malignancy, many surgeons are now questioning whether it is appropriate to apply these techniques to malignancy (Clair *et al,* 1993; Champault *et al,* 1994; Cotlar *et al,* 1996; Downey *et al,* 1996; Dixit *et al,* 1997).

Recent evidence from experimental studies supports the postulate that the insufflation of carbon dioxide gas used to facilitate laparoscopic exposure, may lead to tumour dissemination to laparoscopic port sites (Jones *et al,* 1995). In the earlier study reported (Chapter 3) utilising the DA rat model with an implanted abdominal malignancy, it was demonstrated that tumour laceration during laparoscopy with CO_2 pneumoperitoneum increased the incidence of metastasis to surgical wounds by five-fold, compared to an identical procedure performed at open surgery. When this study was repeated using gasless laparoscopic exposure, the incidence of wound metastases was similar to that associated with laparotomy (Chapter 4).

To investigate port site metastases further, an alternative experimental model has been developed to investigate patterns of tumour implantation. In this study a tumour cell suspension was introduced into the peritoneal cavity at both open and laparoscopic surgery, instead of the laceration of a pre-existing tumour. This removed the potential variability in the number of tumour cells liberated into the peritoneal cavity when using the laceration technique.

Materials and Methods

Thirty six syngeneic Dark Agouti (DA) rats, weighing between 240 and 280 g (median 250), were used for this study. A 200 µl volume of a suspension containing 1 x 10^7 viable adenocarcinoma cells was prepared using a standardised technique by homogenising fresh tumours resected from the carrier rats.

The tumour cell suspension was prepared according to the method described earlier. The final concentration was adjusted to give 1 x 10^7 cells in 200 µl of sterile PBS. This was then introduced into left upper quadrant of the peritoneal cavity of all animals at the commencement of one of three surgical procedures. The tumour cell dose was determined by a pilot study described earlier (Chapter 5.1).

Under general anaesthesia, using a combination of halothane and nitrous oxide supplemented with oxygen via a close fitting mask, thirty six animals underwent one of three different surgical procedures (12 in each group). The respiratory status of each animal was closely monitored and the procedures were performed under sterile operating conditions.

Laparotomy

Twelve rats underwent a conventional laparotomy through a 3 cm long midline abdominal incision. The tumour cell suspension was introduced slowly into the left upper quadrant of the peritoneal cavity through an 20 gauge intravenous cannula. Care was taken not to inadvertently contaminate the abdominal wound or any other site with

the suspension. The abdominal cavity was then left open for 45 minutes, before being closed with 3-0 Prolene sutures.

Laparoscopy using carbon dioxide pneumoperitoneum

Twelve rats underwent laparoscopy facilitated by conventional CO_2 pneumoperitoneum. Pneumoperitoneum was initially established using the standardised technique described earlier and using the standardised equipment. CO_2 gas was insufflated at a flow rate of 0.2 L/ min and a maximum pressure of 4 mmHg.

A 20 gauge intravenous cannula was next introduced through the left upper quadrant abdominal wall. The tumour suspension was introduced slowly through this cannula, before sealing the cannula to prevent gas leakage. An 18 gauge intravenous cannula was then introduced into the abdominal cavity through the left lower quadrant of the abdominal wall and left 'open' to air. This acted as a venting cannula, ensuring a constant circulation of CO_2 through the peritoneal cavity. Pneumoperitoneum was maintained for 45 minutes, before removing all ports and closing the wounds with 3-0 Prolene sutures.

Laparoscopy using a gasless technique

In this group, 12 rats underwent laparoscopic surgery using an identical protocol to the conventional laparoscopy group, except CO_2 insufflation was omitted and a gasless technique was used. A Veress needle without insufflation was still used for the introduction of the first trocar. A working space in the abdominal cavity was maintained by suspending the anterior abdominal wall from a wire frame, using multiple 3-0 silk sutures passed through the abdominal wall skin and anchored to the frame. A 20 gauge and an 18 gauge intravenous cannula were introduced separately into the left upper quadrant and the left lower quadrant respectively and the tumour cell suspension was slowly introduced into the peritoneal cavity through the left upper quadrant cannula. This cannula was then closed to air and the left lower quadrant port was left open to atmospheric air. All ports were left in place for 45 minutes while the

abdominal wall remained suspended to ensure an adequate laparoscopic working space. The ports were then removed and the wounds were closed with 3-0 Prolene sutures.

The animals recovered from all surgical procedures and were housed in the laboratory for six days. Food and water consumption was normal within 24 hours of the surgical procedures. Six days after tumour implantation all animals were killed and an autopsy examination was performed. The abdomen was opened, examined for tumour deposits and the tumour implantation pattern assessed using a standardised scoring system. The abdominal cavity was divided into six sectors (Figure 5.1) and the presence and pattern of tumour implantation documented for each sector. The density of tumour implantation was scored according to the peritoneal cancer index as described in Section 5.1 (Eggermont *et al,* 1988).

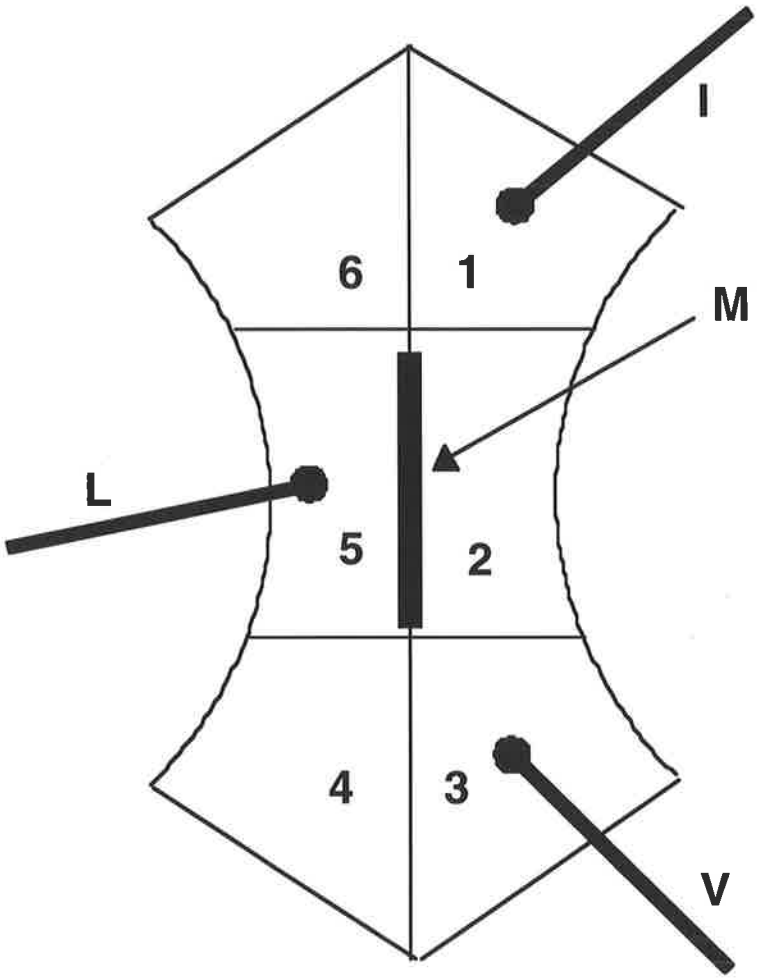
Each sector was therefore scored for the presence or absence of tumour deposits and for tumour density. Representative samples were examined histologically to confirm the macroscopic assessment. All surgical access wounds were examined for macroscopic evidence of tumour implantation.

Statistical analysis

Fisher's exact test was used for the analysis of data sets expressed by 2 x 2 contingency tables and the Chi-squared test was used for the analysis of larger contingency tables.

FIGURE 5.1

ABDOMINAL CAVITY DIVIDED INTO SIX SECTORS



I - Injection cannula

V - Venting cannula

L - Laparoscopic cannula

M - Midline incision

Results

All animals recovered quickly from the operative procedure and none showed adverse effects during the ensuing 6 day period. Tumour deposition and growth was present within the peritoneal cavity of all rats. The pattern of tumour implantation seen in each experimental group is summarised in Table 5.1. In the laparoscopy with CO_2 group, tumour implantation was common in all sectors of the peritoneal cavity. In both the laparotomy and gasless laparoscopy groups, tumour implantation was uncommon on the right side of the abdominal cavity. Tumour implantation in the right upper (sector 6) and right lateral (sector 5) sectors was significantly more likely in rats undergoing insufflation with CO_2 gas. These sectors were distant from the site of introduction of the original tumour cells and were to some extent physically separated from the introduction site by the vertical orientation of the rat colon. Tumour implantation in the laparotomy and gasless laparoscopy groups tended to follow a route of dependent drainage along the rat's 'paracolic gutter'.

TABLE 5.1

EVIDENCE OF MACROSCOPIC TUMOUR

IN EACH SECTOR OF THE PERITONEAL CAVITY

(12 animals per group)

Sector	Laparotomy	Laparoscopy with CO_2	Gasless Laparoscopy	P value *
1	12	12	12	P = 1.0
2	10	11	9	P = 0.55
3	8	11	8	P = 0.26
4	3	8	5	P = 0.12
5	0	12	2	P < 0.0001
6	0	10	1	P < 0.0001

* = Statistical comparisons by the Chi-squared test.

The analysis of the surgical wound sites is summarised in Table 5.2. Tumour implantation occurred in the midline laparotomy wound of only one animal. Probably because of direct contamination with tumour cells, implantation occurred in all laparoscopic port wounds used for introducing the tumour cell suspension in all animals. However, implantation was significantly less likely at both the laparoscope introduction site (2/ 12 versus 12/ 12, $P < 0.0001$) and the venting cannula site (5/ 12 versus 11/ 12, $P = 0.027$) in rats undergoing gasless laparoscopy compared to conventional laparoscopy with CO_2 insufflation.

TABLE 5.2

MACROSCOPIC TUMOUR EVIDENT IN SURGICAL WOUNDS

(12 animals per group)

Wound	Laparotomy	Laparoscopy with CO_2	Gasless laparoscopy	P value*
Midline	1	N/A	N/A	
Injection port	N/A	12	12	P = 1.0
Venting port	N/A	11	5	P = 0.027
Laparoscope port	N/A	12	2	P< 0.0001

N/A = No applicable wound in this group.

* = Statistical comparisons by Fisher's exact test.

Overall tumour grading is summarised in Table 5.3. Significantly more sectors exhibited tumour implantation in the group undergoing laparoscopy with CO_2 insufflation. The majority of rats exhibited confluent or moderate tumour growth in the sectors in which tumour implantation was evident.

TABLE 5.3

SUMMARY OF TUMOUR GRADING IN SECTORS OF PERITONEAL CAVITY

(12 animals x 6 sectors per group)

Number Of Sectors Involved

Grade	Laparotomy	Laparoscopy with CO_2	Gasless Laparoscopy
0	39	8	35
I	4	3	4
II	4	23	20
III	25	38	13

Discussion

The studies using the solid model reported in a previous chapter (Chapter 3) demonstrated a five-fold increase in the incidence of port site metastasis following laparoscopic surgery using CO_2 insufflation. However, when CO_2 insufflation was eliminated by using a gasless laparoscopic technique, the port site metastasis rate was significantly less (Chapter 4), resembling the incidence of wound metastasis following laparotomy in this model. A similar three-fold increase in the incidence of wound metastasis was reported by Jones *et al* (1995) in an experimental model which used a tumour cell suspension in a hamster. On the other hand, others have argued that laparoscopy is in fact beneficial and reduces the likelihood of tumour implantation (Bouvy *et al,* 1996a). However, because they introduced their tumour cell suspension at the conclusion of surgery, not at the beginning, they failed to adequately investigate the effects CO_2 insufflation. Only the beneficial effect of reduced immune suppression during laparoscopy was investigated, a phenomenon which has also been documented by other researchers.

The current study sought to investigate the potential effect of laparoscopy and CO_2 insufflation on tumour implantation patterns. Rather than investigate whether laparoscopy influences the likelihood of tumour implantation in individual animals, a higher dose of tumour cells than that used by other workers was introduced intraperitoneally, to ensure that tumour implantation occurred in all animals studied. In this study a similar number of free tumour cells was introduced in a standardised fashion into the same site in the peritoneal cavity, enabling the variable of laparoscopy with or without CO_2 insufflation to be investigated. Whilst the number of free cells in the rats' peritoneal cavity may be in excess of the usual quantity present in clinical settings, the study does provide information about the possible effect of clinical laparoscopic procedures on free tumour cells present in, or liberated into, the human peritoneal cavity at the time of surgery.

The implantation pattern of tumour in the peritoneal cavity of rats undergoing laparoscopy with CO_2 insufflation was significantly different from that observed following laparotomy or gasless laparoscopy. Insufflation resulted in tumour redistribution and spread to all sectors and all port sites in the majority of animals. In contrast tumour implantation in the other two groups was predominantly on the left side of the abdominal cavity i.e. near the site of introduction of the tumour suspension. Similar patterns were seen for the spread of tumour to the surgical access wounds. This suggests that the insufflation of CO_2 gas during laparoscopy leads to more widespread tumour deposition and growth, compared to gasless laparoscopy and laparotomy. However, this study has not clarified whether this phenomenon is due solely to physical effects of the insufflating gas, or whether metabolic effects (due to possible CO_2 induced alterations in tumour metabolism, or the pH environment of the peritoneal cavity) are involved. This requires further investigation.

The results from this study add weight to the existing evidence, which suggests that wound metastases and tumour spread is more likely to occur following laparoscopic procedures for malignancy when using CO_2 insufflation.

CHAPTER 6

FREE TUMOUR CELL AND LAPAROSCOPIC GAS LEAK

6.1. The Effect of Gas Leak through an Open Wound during CO_2 Pneumoperitoneum on the Implantation of Tumour.

Background

Studies using the DA rat model with the solid tumour and free cell model demonstrated that the presence of CO_2 pneumoperitoneum increases the likelihood of tumour spread to port sites. However, it is inconclusive from experimental work so far if this is due to the effect of gas physically moving the cellular matter around, or if it is due to increased deposition of tumour cells on laparoscopic instruments & ports, and direct implantation. Both these mechanisms have been postulated (Hewett *et al,* 1996; Hubens *et al,* 1996).

There is experimental evidence that tumour cells can grow preferentially in injured tissue and that malignant cell growth is facilitated in areas of high cell proliferation (Murthy *et al,* 1991). The local release of growth factors may also enhance the development of tumour metastases (Roberts *et al,* 1988; Savalgi & Rosin, 1996). The port site wound may provide such an environment. However, this does not provide a satisfactory explanation for port site incidence of tumour metastases. If the metastases were only due to the presence of a wound, then the incidence of port site metastases should be the same in laparotomy, laparoscopy with gas and without gas. There is preliminary evidence to suggest that cellular matter may be physically dispersed by circulating gas, as findings from an earlier study have demonstrated clumps of cells in the smoke produced by electro coagulation during laparoscopic surgery (Lauroy *et al,* 1994). At the same time, other investigators have reported

significant contamination of laparoscopic instruments and ports, but did not find a significant number of cells in the gas escaping through the ports (Doudle *et al,* 1996).

It has been postulated that gas leaks around ports during laparoscopic surgery produce a chimney effect or a venturi phenomenon, which is a high pressure - high flow situation of gas (Hubens *et al,* 1996). It has been speculated that this effect can transport particulate or cellular matter, which are trapped in the wounds through which the gas is leaking (Jones *et al,* 1995).

If the circulating gas does transport cells, it would be logical to observe an increase in the incidence of port site metastases when CO_2 pneumoperitoneum is induced, compared to the gasless procedure.

Aim of the study

The following study was conducted to clarify this issue. In this experimental study, the free cell model was used to study the effect of gas alone venting out through a small hole made in the abdominal wall, without the presence of a cannula. The hypothesis being that if port site metastases are primarily due to the gas depositing tumour cells on the cannula and not by the gas physically dispersing the cells, then there should be a significant increase in the tumour deposition around port sites with cannulae than in the open wounds without cannulae.

Materials and Methods

Twelve DA rats were used for this study. A group of six rats underwent laparoscopy with CO_2 insufflation, while another group of six rats underwent laparoscopy without CO_2 insufflation or "gasless laparoscopy". All animals underwent the procedures under closely monitored anaesthesia, using a combination of halothane, oxygen, supplemented by nitrous oxide.

Free tumour cell suspension from harvested tumours were prepared by a standardised technique described in the Chapter 2. The final concentration was adjusted to give 1 x 10^7 cells in 200 µl of sterile PBS. This was then introduced into left upper quadrant of the peritoneal cavity of all animals at the commencement of one of three surgical procedures.

Laparoscopy without cannula and venting hole using CO_2 insufflation

Six rats underwent laparoscopy by a modified technique to what was used earlier. Pneumoperitoneum was initially established using a Veress needle sited through a small stab incision to the right of the umbilicus. A disposable mini laparoscopy cannula was introduced to provide access for a mini laparoscope. A 20 G cannula was introduced into the left upper quadrant for the injection of cell suspension. The Veress needle and a fresh cannula was inserted through a stab incision on the opposite side to create a venting hole. The Veress needle and cannula were removed and insufflation was commenced, to ensure that the venting hole was venting CO_2 out, without losing the pneumoperitoneum. The venting hole was then closed by pinching it with a haemostat. The tumour cell suspension was slowly introduced into the peritoneal cavity through the cannula in the left upper quadrant, under vision so as not to inject into any intraperitoneal organs. The injecting cannula was then sealed. This technique was similar to the one described in the previous study.

The venting hole was then opened and CO_2 gas allowed to vent through, while the pneumoperitoneum was maintained. The pneumoperitoneum was maintained for 30 minutes. At the end of 30 minutes the insufflation was stopped, the insufflating cannula removed and the incision closed with sutures.

Laparoscopy using a gasless technique

Six rats underwent a similar procedure to that performed described above, but without the use of CO_2 pneumoperitoneum. A Veress needle with a trocar was introduced without CO_2 insufflation. The intraperitoneal position was ensured by

injecting a small amount of air. A similar method was used to create a venting hole in the opposite side of the abdomen. A working space was then created in the abdominal cavity by suspending the anterior abdominal wall from a wire frame, using a series of sutures passed through the abdominal wall skin and anchored to a frame.

The cannula was left open to atmospheric air. Tumour cell suspension was slowly introduced into the peritoneal cavity, under vision through the laparoscope. The cannula on the left side of the abdomen was removed after 30 minutes and the incisions were closed with sutures.

All the animals recovered from the surgical procedures. They were allowed food and water freely. Six days after the tumour cell injection, all the animals were killed and an autopsy examination was performed. The abdomen was opened and examined for tumour deposits especially in the port site wound and also for any evidence of spread of tumour outside the abdominal wall. The tumour implantation pattern was assessed using the peritoneal cancer index as described in Chapter 5.1 (Eggermont *et al,* 1988). This scoring was assessed by naked eye examination.

The venting port sites were examined to detect if there was tumour in the vicinity of the wound; in the wound itself i.e. in the abdominal musculature; and for evidence of spread outside the abdominal wall, i.e. tumour in the subcutaneous tissue around the ports.

Statistics

The data from this study was analysed using the Chi-squared test or Fisher's exact test.

Results

At autopsy all the animals had tumour in the peritoneal cavity. In the gasless laparoscopic group, the grade of tumour growth was less in all quadrants, they were grade 2. Three of the animals had tumour around the port site which was left open. There was no tumour in the abdominal musculature at the site of the wounds. There was also no tumour in the subcutaneous tissue around the port sites.

TABLE 6.1

NUMBERS OF ANIMALS WITH GRADE 3 MACROSCOPIC TUMOUR EVIDENT IN EACH SECTOR OF THE PERITONEAL CAVITY

(6 animals per group)

Sector	Laparoscopy with CO_2	Gasless Laparoscopy	P value *
1	4	1	P = 0.2424
2	4	1	P = 0.2424
3	5	1	P = 0.080
4	5	0	P = 0.0152
5	5	0	P = 0.0152
6	5	0	P = 0.0152

* = Statistical analysis by Fisher's exact test

TABLE 6.2

NUMBER OF ANIMALS WITH MACROSCOPIC TUMOUR EVIDENT IN SURGICAL WOUNDS

(6 animals per group)

Wound	Laparoscopy with CO_2	Gasless Laparoscopy	P value *
Laparoscope port	6	2	NS
Venting port	6	3	NS
Tumour muscle	6	0	P = 0.0022
Tumour subcutaneous tissue	5	0	P = 0.0152

* = Statistical comparisons by Fisher's exact test.

Tumour muscle - These animals had tumour within the musculature of the abdominal wall.

Tumour subcutaneous tissue - These animals had tumour in the subcutaneous tissue plane outside the abdominal cavity around the port site.

TABLE 6.3

SUMMARY OF TUMOUR GRADING IN SECTORS OF PERITONEAL CAVITY

Number Of Sectors Involved (6X6 sectors per group)

Grade	Laparoscopy with CO_2	Gasless Laparoscopy	P value
Grade < III	8	33	P = 0.0001
Grade III	28	3	

* = Statistical comparisons by the Fisher's exact test.

In the group that underwent laparoscopy with CO_2, all six rats developed intra peritoneal tumour. The tumour spread to all the sectors. All the sectors had grade 3 tumours. All the animals had tumour around the venting wound and in the abdominal musculature. This finding was not seen in the other studies so far and in the earlier studies, tumours were around the port site on the peritoneal aspect. Five of the six rats had tumour in the subcutaneous plane outside the venting wound. This finding too was unique to this study, as in all the previous studies there was no exuberant growth of the tumour in the subcutaneous tissue around the port sites, on the outer aspect of the abdominal wall.

Discussion

In the previous study using the free cell model, described in Chapter 5, it was demonstrated that CO_2 pneumoperitoneum causes an increase in the distribution and growth of free tumour cells injected intraperitoneally. Thus, CO_2 pneumoperitoneum had an adverse impact on the implantation and dissemination of tumour in this animal model. However, it unclear if this was due to the physical effect of the gas, or due to other metabolic and immunologic changes produced by CO_2 in the peritoneum. Some workers investigating the same phenomena in experimental models have postulated CO_2 gas circulating cells around the peritoneum (Jones *et al,* 1995; Jacobi *et al,* 1997b; Texler *et al,* 1997), while others have suggested that cells are being deposited on cannulae, which then contaminate the ports (Jacobi *et al,* 1995; Hewett *et al,* 1996).

This study was performed with the hypothesis that:

a. if the occurrence of port site metastases is mainly due to contaminated cannulae (Cook & Dehn, 1996; Thomas *et al,* 1996), then the incidence of port site metastases should be less when there are no cannulae in the wound.

b. if gas is physically transporting the tumour cells, then the occurrence of tumour in a wound through which gas is venting out should be greater, than when no gas is venting out.

In this study, there was a significant increase in the incidence of tumour depositing in the abdominal wall musculature at the site of the venting wound (0/ 6 vs. 6/ 6) and increased incidence of subcutaneous tumour (0/ 6 vs. 5/ 6) in the laparoscopy with CO_2 group.

It has been postulated that the presence of high flow, results in gas leaks around the cannulae or port sites produce by a local venturi effect and this leads to deposition of cells around the port site (Allardyce *et al,* 1996; Bouvy *et al,* 1996c).

Any tumour spread outside the wound could only be explained by the tumour cells being carried by the venting gas and being deposited in the subcutaneous space, by the trap door mechanism between the opening in the abdominal muscle and the skin.

These results give support to the argument that gas by itself can cause increased deposition of tumour in wounds, even in the absence of cannulae or instruments. This also lends credibility to one of the mechanisms proposed i.e. the chimney effect (Hubens *et al,* 1996).

There is evidence that tumour cells implant in areas of trauma and possibly ischaemia (Murthy *et al,* 1991). Port sites used in laparoscopic surgery may be locations of ischaemia (Baker *et al,* 1989; Wu & Mustoe, 1995). Growth factors produced by wounds have been shown to enhance not only wound healing, but also the invasion and growth of cancer cells (Eggermont *et al,* 1988; Roberts *et al,* 1988; Murthy *et al,* 1989). Ischaemia reduces local defence mechanisms, rendering the ischaemic site vulnerable to tumour implantation and growth (Alexander & Altemeier, 1963; Schilling *et al,* 1997).

Most of the reported cases of abdominal wall metastases developed tumour in the subcutaneous tissue close to the trocar incisions (Walsh *et al,* 1993; Jacobi *et al,* 1995). Carbon dioxide has been demonstrated to produce a significant degree of acidosis in the peritoneal as well as the subcutaneous tissue (Volz *et al,* 1996). This may be explained by a high solubility of carbon dioxide in subcutaneous tissue (Jacobi *et al,* 1997a). The results of this study showed almost all the animals developed tumours in the subcutaneous tissue around the venting port. This data suggests that insufflation of carbon dioxide is an important factor, which promotes port site and subcutaneous metastases.

CHAPTER 7 FREE TUMOUR CELL WITH GAS AND GASLESS LAPAROSCOPY

7.1. Movement of Tumour Cells following Laparoscopy, with and without CO_2.

Introduction

The previous studies using the solid tumour model (Chapter 3) in the DA rat model, demonstrated an increased incidence of wound metastasis following laparoscopy with CO_2 insufflation. This adverse effect can be minimised by gasless laparoscopic exposure techniques (Chapter 4), suggesting that CO_2 insufflation during laparoscopy promotes wound metastasis. In the free cell model (Chapter 5), there was added evidence that there is a significant increase in the distribution, implantation and growth of intraperitoneal tumour with CO_2 insufflation. Other studies using small animal models have demonstrated an increased incidence of wound metastasis following laparoscopy in the presence of intra-abdominal malignancy (Hewett *et al,* 1996). While studies using large animal models have demonstrated that laparoscopy in the presence of free intraperitoneal tumour cells can lead to the contamination of laparoscopic instruments and trocar shafts with tumour cells (Allardyce *et al,* 1996; Hewett *et al,* 1996; Thomas *et al,* 1996).

Different mechanisms have been postulated which may explain this phenomenon (Nduka *et al,* 1994; Thomas *et al,* 1996). Wound contamination could result from direct contact between laparoscopic instruments or trocars and a tumour, resulting in wound contamination when the instruments are withdrawn during the laparoscopic procedure, or when the trocars are removed at the end of the operation (Allardyce *et al,* 1996; Thomas *et al,* 1996). Alternatively, tumour cells might be aerosolised by the insufflation gas and then transported by the gas to the laparoscopic

wounds, instruments and trocar shafts, resulting in wound contamination without direct physical contact with tumour bearing tissue. In the previous study (Chapter 6), using the venting hole in the presence of free intraperitoneal tumour cells, results indicated that the insufflating CO_2 gas may be able to transport cells directly to the wound through which there is a gas leak.

Aim of this study

The aim of this study was to investigate if a large gas leak could transport viable cells. The viability of the cells was tested by circulating the venting CO_2 through a recipient rat.

Materials and Methods

Preparation of viable adenocarcinoma suspension

Viable tumour cell suspensions of DAMA were prepared from harvested tumours by a standardised method described earlier (Chapter 2). The final concentration was adjusted to give 1 x 10^8 viable cells in 1.0 ml of sterile PBS. This was then introduced into the epigastric region of the peritoneal cavity of all animals at the commencement of each experiment.

Under general anaesthesia, using a combination of halothane and nitrous oxide supplemented with oxygen via a close fitting mask, twenty four male syngeneic immunocompetent DA rats underwent one of two different surgical procedures. The respiratory status of each animal was closely monitored and the procedures were performed under sterile operating conditions.

The model consisted of "donor" rats which underwent either a laparoscopic procedure facilitated by conventional CO_2 pneumoperitoneum or a gasless laparoscopic procedure. A "recipient" rat was connected to the "donor" rat by a 14 gauge plastic tube inserted into the abdomen. This cannula arrangement provided a conduit for carbon dioxide insufflation gas to be vented from the donor rat through to the peritoneal cavity of the recipient rat, and subsequently to the atmosphere in the insufflation group. The

same cannula arrangement was used to study a control group using the gasless laparoscopic technique.

Six pairs of rats underwent a laparoscopic procedure facilitated by conventional CO_2 pneumoperitoneum. Pneumoperitoneum was initially established in both the donor and recipient rats using a Veress needle placed through a small stab incision in the right antero-lateral abdominal wall of each rat. A disposable mini-laparoscopy cannula was introduced the needle to provide access for a mini-laparoscope attached to a conventional laparoscopic camera. Under laparoscopic vision, a second mini-laparoscopy cannula was introduced through the left lower quadrant of the anterior abdominal cavity of the donor rat only. This cannula was then removed and the end of a piece of 14 gauge plastic tubing (7 cm length) was introduced through the wound so that 5 mm lay within the peritoneal cavity. A purse string suture was placed around the cannula to achieve a gas tight seal.

Under laparoscopic vision, an 18 gauge cannula was introduced through the antero-lateral aspect of the left lower abdominal wall of the recipient rat. This was then left open to allow subsequent venting of the insufflation gas. Insufflation was disconnected from the recipient and the mini-laparoscopy cannula used for initial laparoscopy was removed. The other end of the plastic tubing, placed earlier in the donor rat, was introduced through the wound and secured with a purse string suture (Figure 7.1). Insufflation was commenced through the mini-laparoscopy cannula remaining in the donor. The cannulae and tubes were checked during insufflation to ensure that gas leaked through the open cannula in the recipient rat only.

A 20 gauge intravenous cannula was next introduced through the abdominal wall in the epigastric region of the donor rat and 1.0 ml of the DAMA cell suspension containing 1×10^8 cells was introduced slowly through this cannula, before sealing the cannula to prevent subsequent gas leakage. CO_2 gas was insufflated at a flow rate of 0.2 L/ min at a maximum pressure of 6 mmHg for 30 minutes, achieving a constant circulation of carbon dioxide through the peritoneal cavities of the two animals. To

prevent direct contamination of the cannula with tumour cells or siphoning of fluid from the donor to the recipient, care was taken to ensure that the cannula tips in both rats did not come into contact with the abdominal contents or any intra-abdominal fluid. The insufflation phase was terminated by disconnecting the plastic tubing from the recipient, before removing the other 18 gauge cannula. All wounds were closed with 3-0 Prolene sutures.

The donor rats were killed 6 days after surgery, and an autopsy examination was performed to confirm tumour growth within the peritoneal cavity of these rats and therefore the viability of the tumour cell suspension. The recipient rats were housed in the laboratory for 21 days before being killed. At autopsy examination, the abdomen was opened and examined for tumour deposits. All of the surgical access wounds were examined for evidence of tumour implantation. Histological examination using haematoxylin and eosin staining of formalin-fixed, wax-embedded tissue was used to confirm the macroscopic assessment.

A further 6 pairs of rats underwent laparoscopic surgery using an identical protocol to the study using carbon dioxide insufflation, except insufflation was omitted and a gasless technique was used. A Veress needle, without insufflation, was used for the introduction of the first trocar in each rat. A working space in the abdominal cavity of each rat was maintained by suspending the anterior abdominal wall from a wire frame.

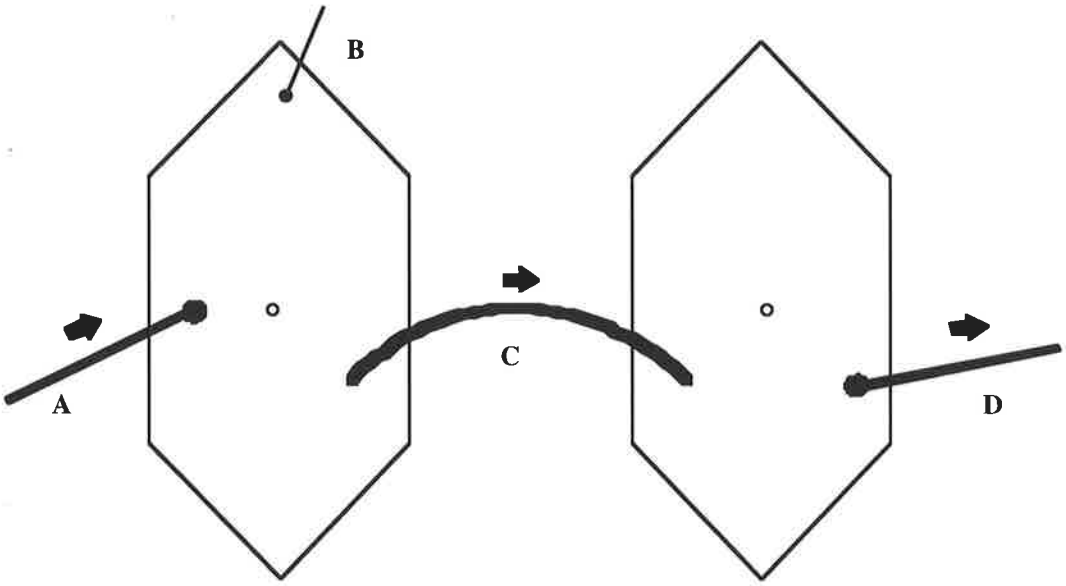
The experiment was conducted in a similar fashion to the insufflation study, with autopsy examination of the donor rat at 6 days (to confirm the viability of the tumour cell suspension) and the recipient rat at 21 days.

Statistics

Fisher's exact test was used for the analysis of the data sets expressed as 2 x 2 contingency tables for this experiment.

FIGURE 7.1

ARRANGEMENT FOR THE FLOW OF FREE TUMOUR CELLS FROM THE "DONOR" RAT TO THE "RECIPIENT" RAT



"Port" placement for experiment: Arrows indicate the direction of gas flow during CO_2 insufflation.

A = primary insufflation port and mini-laparoscope port

B = 20 gauge cannula for the introduction of tumour suspension

C = 14 gauge plastic tubing

D = 18 gauge venting cannula

Results

Extensive tumour growth was evident throughout the peritoneal cavity of all donor rats, irrespective of surgical technique and at all wound sites. This confirmed the viability of the cell suspension in all instances. Nodular tumour metastases were found around the site of both the venting port and the in-flow tubing site in 5 out of 6 of the recipient rats, in the group which underwent carbon dioxide insufflation (Table 7.1). Tumour was not found at any other sites within the abdominal cavity of the recipient rats.

None of the recipient rats in the gasless laparoscopy group developed metastatic tumour, either around the venting cannula site (P = 0.015, Fisher's exact test), or elsewhere.

TABLE 7.1

TUMOUR METASTASES IN RECIPIENT RATS

	Laparoscopy with CO_2	**Gasless laparoscopy**
Venting port	5 / 6	0 / 6

P = 0.015 (Fisher's exact test)

Discussion

Previous experimental studies described in earlier chapters have demonstrated an increased incidence of metastasis to trocar wounds following laparoscopy in the presence of intra-abdominal malignancy. This has also been demonstrated in studies conducted by other investigators (Jones *et al,* 1995). Recently other studies have demonstrated that insufflation is a major contributing factor in the development of laparoscopic wound metastasis (Bouvy *et al,* 1996b).

In the current study the presence of wound metastases in the recipient rat occurred in most of the rats undergoing carbon dioxide insufflation and none of the rats in the gasless laparoscopy group, providing additional evidence that insufflation is essential for the promotion of wound metastases. However, it could be argued that the study outcomes were due to siphoning of fluid between the donor and recipient rats. Care was taken during the studies to prevent this from happening. Furthermore, if siphoning of fluid occurred, then metastases would be expected in the recipient rat in the gasless laparoscopy group and metastases would have been more prominent around the in-flow port wound in the recipient rats. However in the study, tumour nodules of equal size occurred at both the in-flow and out-flow port wounds in the recipient rats undergoing insufflation. A larger inoculum of tumour cells was introduced into the donor rats in these studies than that used in earlier studies, described in the earlier chapters (Chapters 5, 6). This was to maximise the likelihood of metastases developing in the wounds in the recipient rats and to optimise the likelihood of demonstrating differences in tumour implantation patterns between the insufflation and gasless laparoscopy groups. Similarly, because of the possible small tumour load in the recipient rats, a longer latency period of 21 days (previously 7 days) was used to maximise the likelihood of tumour growth being clinically evident in the recipient rats.

Studies by Whelan *et al* however, have revealed conflicting results (Whelan *et al,* 1996). Their studies used a quantity of tumour cells which was 1000 times smaller than that used in the current studies and insufflation was continued for only 10

minutes. Outcome differences may therefore reflect a dose response and time dependent relationship. The initial cell suspension studies performed with the free tumour cell, suggested that tumour implantation patterns are influenced by the number of viable cells introduced into the peritoneal cavity (Chapter 5.1) and it is possible that the duration of exposure to the laparoscopic environment may also be important.

The current study assessed a laparoscopic environment which may be different to that thought to exist during clinical laparoscopic cancer surgery. The CO_2 leak of 0.2 L/ min in the rat model, when extrapolated to clinical surgery, represents a gas leak of 10 to 15 L/ min. Also, a large number of tumour cells were introduced into the peritoneal cavity and no operative intervention was performed. However, it should be remembered that during clinical laparoscopic surgery, high volume gas leaks do occur during some procedures and the inadvertent liberation of large numbers of tumour cells into the peritoneal cavity can occur. Whilst it is possible that careful laparoscopic technique may avoid the liberation of tumour cells, this cannot be guaranteed pre-operatively and tumour cell spillage remains a real possibility. If the laparoscopic technique is combined with CO_2 insufflation and a significant gas leak, then the necessary environment for clinically important port site metastases may be created.

In this different experimental model, there is further support that laparoscopy with carbon dioxide insufflation transports tumour cells to laparoscopic access wounds and results in the growth of metastases. This effect appears to be prevented by the use of gasless laparoscopy (Chapter 4). The precise mechanism for this phenomenon however, is not clear. Whether the differences demonstrated are due to: the mechanical effects of insufflation; a metabolic effect specific to CO_2 gas; or a combination of both, will require further elucidation by using other gases. While this study confirms the need for caution when applying laparoscopic techniques to malignancy in humans, further investigations of gasless laparoscopy and non-CO_2 gases in clinical studies for malignancy is indicated.

CHAPTER 8 RADIO-LABELLED FREE TUMOUR CELLS

8.1. Radio-labelled Cells: a Model for Studying Cell Dispersion.

Background

There is no agreement about the mechanism responsible for the incidence of port site recurrence following laparoscopic surgery. Many contributing factors have been postulated, some of these relate to the general factors of surgery in the presence of malignancy (Savalgi & Rosin, 1996), such as surgery induced immunosuppression (Jones *et al,* 1995), tumour cell spillage into surgical field (Cirocco *et al,* 1994) and tumour cell adherence to areas of tissue trauma (Jacobi *et al,* 1995). Others relate to factors more specific to the practice of laparoscopic surgery, such as direct contact between contaminated laparoscopic instruments or trocars and wounds during the laparoscopic procedure, or when the trocars are removed at the end of the operation (Cook & Dehn, 1996). Alternatively, tumour cells aerosolised by the insufflated gas may be transported to the laparoscopic wounds, instruments and trocar shafts, resulting in wound contamination (Hubens *et al,* 1996). Thus wound implantation may be an indicator of wider tumour cell dissemination, or it may be a local phenomenon.

The previous studies reported in Chapters 6 and 7 have demonstrated that there is a definite influence of gas on the transport of cells within and outside the peritoneal cavity. However, it is unknown what quantity of cells are moved during this process. Although survival studies with the DA rat model, described in the previous chapters, have demonstrated that CO_2 can cause increases in implantation and growth of tumour cells in the peritoneal cavity, it is unclear what quantity of cells are transported and whether there is a difference in the distribution of cells during surgical procedures. The present model was developed to investigate the pattern and enumerate the distribution

of cells immediately following laparotomy, laparoscopy with CO_2 and gasless laparoscopy.

Radio labelling technique

Viable DAMA cells were prepared using a standardised technique. Subcutaneous tumours of approximately 5 g were excised from carriers, were homogenised and the crude debris removed. The cell suspension was next centrifuged four times in 10 volumes of PBS at 400g, each for 1 minute. The cell viability was assessed and the final concentration of the suspension was adjusted to give 1 x 10^8 viable cells in 1 ml of sterile PBS. These cells were then radio-labelled with ^{51}Cr-sodium chromate 1 MBq, 0.6 µg Cr ions (CRIM4, Radio-isotopes Australia). The cell suspension was incubated for 60 minutes with ^{51}Cr and the cells were washed five times in sterile PBS. Greater than 75% incorporation of the ^{51}Cr into the tumour cells was obtained in all instances. These cells were introduced into the abdominal cavity and the effect of gas insufflation was studied by measuring the radio activity (Iverson, 1973).

8.2. A Study of Cell Dispersion in the Venting Gas Model.

Introduction

An earlier study reported in this work using the DA rat model, has demonstrated an increased incidence of wound metastases following laparoscopy with CO_2 insufflation (Chapter 3). This adverse effect can be minimised by the gasless laparoscopic exposure technique (Chapter 4), suggesting that CO_2 insufflation during laparoscopy plays a crucial role in the development of wound metastases. However, the exact mechanism by which CO_2 insufflation produces this effect is not clear. There is evidence to indicate that the free cells can be transported to the port site during high pressure leaks, like that which occurs during desufflation or accidental dislodgement of a port (Whelan *et al,* 1996).

Aim of the study

To investigate the potential for CO_2 gas to transport tumour cells during large high pressure leaks.

Materials and Methods

DA rats weighing an average of 240 g were used. All rats underwent the surgical procedures under anaesthesia. Ten DA rats were used for this study.

Laparoscopy with CO_2

Five rats underwent laparoscopy with carbon dioxide insufflation. Laparoscopic exposure was achieved using identical methods to those described in the earlier chapters (Chapter 2). A Veress needle was introduced through a stab incision in the left antero-lateral abdominal wall of each rat and a mini-laparoscopy cannula was used to provide access for the mini-laparoscope (Figure 8.1). A second mini-laparoscope cannula was introduced through the left lower quadrant anterior abdominal wall and then removed. This allowed a 7 cm length of 14 gauge plastic tubing to be introduced through the

wound, 5 mm into the peritoneal cavity. A purse string suture was applied to maintain an airtight seal around the tubing. A 20 gauge intravenous cannula was then introduced through the abdominal wall in the epigastrium.

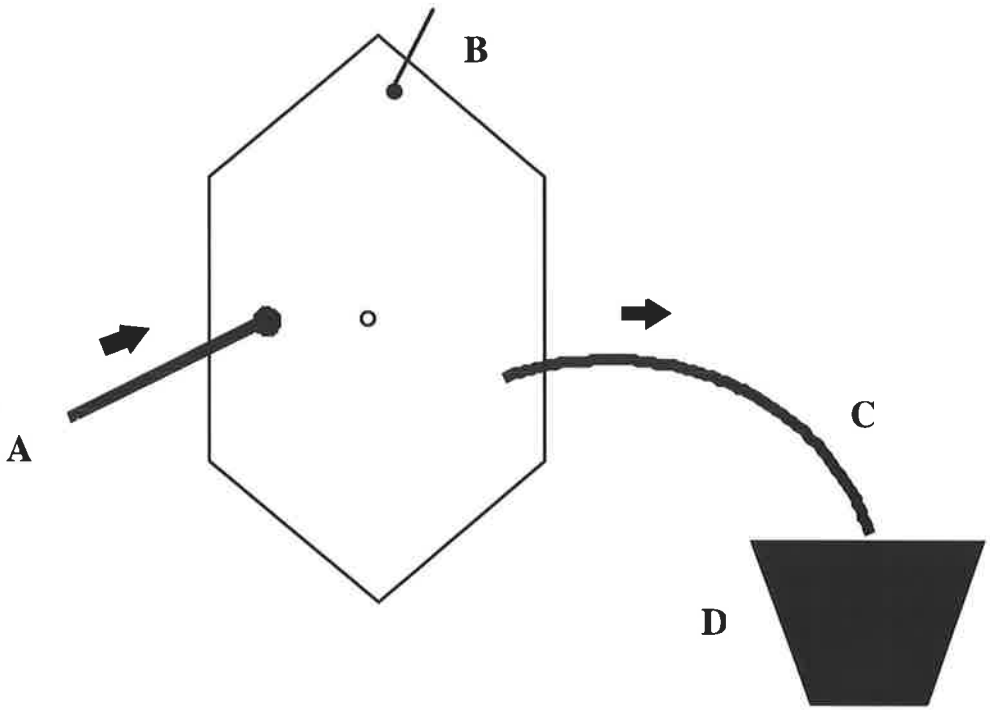
The open end of the plastic tubing was placed in a container of 4 mls of PBS so that the tube opening was 5 mm below the fluid's upper level. Laparoscopic CO_2 insufflation was then commenced and the vented gas was bubbled though the PBS solution. One millilitre of the radio-labelled DAMA cells (approximately 1 x 10^8), pre measured for radioactivity, was then introduced into the peritoneal cavity through the 20 gauge cannula in the epigastrium of each rat. The radio-labelling of the cells was performed according to the method described earlier in this chapter (8.1). The cannula used for intraperitoneal injection was then sealed. The syringe, with the initially drawn dose of radio labelled free cell suspension was measured for radioactivity and the residual activity in the syringe after intraperitoneal injection of the cells was also measured, by passing it through a gamma counter. From these two values, the actual dose injected into the peritoneal cavity was calculated. The radioactivity of the PBS solution was measured before and after the procedure to determine the net radioactivity, which represents the cellular content or cells deposited in the solution. This was calculated as a percentage of the initial dose injected into the peritoneal cavity.

Gasless laparoscopy

Five rats underwent gasless laparoscopy. In the gasless laparoscopic group the experimental conditions were as described in detail earlier in this thesis (Chapter 2.3). The cannula and tubing positions were identical to those used in the CO_2 insufflation group. To prevent siphoning of fluid through the 'venting' tube, care was taken to ensure that the tubing within the peritoneal cavity, did not come into direct contact with the abdominal contents or any intra-abdominal fluid.

FIGURE 8.1

ARRANGEMENT FOR THE INTRODUCTION OF RADIO - LABELLED TUMOUR CELLS INTO THE PERITONEAL CAVITY



"Port" placement for the experiment. Arrows indicate the direction of gas flow during CO_2 insufflation.

A = primary insufflation port and mini-laparoscope port

B = 20 gauge cannula for the introduction of tumour suspension

C = 14 gauge plastic tubing

D = PBS solution.

Measurement of activity in the venting gas

Thirty minutes after the introduction of the radio-labelled DAMA cells the rats were killed and the radioactivity of the PBS solution was determined using a Universal Gamma counter (Compugamma 12820, LKB Wallace) and expressed as a percentage of the total introduced radioactivity of the cell suspension at the commencement of the study. Four millilitres of a control solution of uncontaminated PBS was also counted to assess background radioactivity levels.

Statistics

Data was analysed using the Mann Whitney U-test, to determine differences in the radioactivity levels between the different experimental settings.

Results

The results from the use of radio-labelled tumour cells in the gas and gasless models are summarised in Table 8.1. The recovery of the cells introduced into the peritoneal cavity in the rats undergoing laparoscopy with insufflation was significantly greater than in the rats undergoing gasless laparoscopy (P = 0.008).

TABLE 8.1

RECOVERY OF RADIO-LABELLED CELLS

(all data are median; range)

	Laparoscopy with Insufflation	**Gasless Laparoscopy**	**P value ***
% Recovery of introduced cells	2.71 % (0.41 - 17.32)	0.00 % (0.00 - 0.03)	P = 0.008
Absolute No. of cells recovered	2.7×10^6 $(4.1 \times 10^5 - 1.8 \times 10^7)$	0.0 $(0.0 - 3 \times 10^4)$	

* = Statistical comparisons by the Mann Whitney U test.

Discussion

The aetiology of port site tumour recurrence following laparoscopic surgery is still under debate. Although direct spread is a logical possibility for port site seeding, it does not explain all the reported incidences. Seeding through the removal of instruments contaminated by tumour cells has also been postulated (Hewett *et al,* 1996). The other possible mechanisms which have been proposed, are cells being transported through high pressure leaks around port sites or during desufflation (Whelan *et al,* 1996). Previous studies described in this thesis using the DA rat model have demonstrated that there is an increased incidence of metastases to trocar wounds following laparoscopy, in the presence of CO_2 insufflation (Chapter 3). The gasless technique reduced the incidence to the same level as that of open surgery (Chapter 4). This indicated that the presence of CO_2 gas was crucial. In the studies using the free cells there was evidence to suggest that CO_2 insufflation caused increased dispersion of cells through the venting gas and also increased implantation of tumour cells within the peritoneal cavity (Chapter 5, 6).

In the present study, there was a significant amount of radioactivity measured after laparoscopy with insufflation, indicating that a significant quantity of cells were present in the venting gas, while this was negligible after the gasless laparoscopic technique. The cannula used in this study can be equated to the largest cannula used in the clinical situation. The amount of gas leaking through the cannula was deliberately kept large, which would equate to about 15 L/ min in the human situation. The tumour inoculum used was also large, compared to all the previous studies. This was deliberately chosen to maximise the effect.

In a study by Whelan, using an *in vitro* model, it was found that high flow gas leaks during desufflation are capable of transporting tumour cells (Whelan *et al,* 1996). Thus it is possible that in the presence of a high flow gas leak from the peritoneal cavity and when a significant number of tumour cells are present, the cells could be

transported to a port site and other areas in the peritoneal cavity and cause tumour implantation.

Similar conditions of high gas leak may be present in clinical laparoscopic surgery, when there is excessive movement of instruments through the ports. High pressure leaks can occur when laparoscopic ports are dislodged or during desufflation. Excessive handling of tissue (occurring because of a loss of tactile feedback) can cause a significant increase in the liberation of free tumour cells from the tumour site. A combination of these two factors may result in a large inoculum of free tumour cells dispersing from the peritoneal cavity and may result in conditions favourable for the development of port site metastases.

This study suggests that large gas leaks, in the presence of free tumour cells in the peritoneal cavity, may contribute to the transport of tumour cells to the port sites.

CHAPTER 9

RADIO-LABELLED FREE TUMOUR CELLS DISPERSION

9.1 Pattern of Tumour Cell Dispersion within the Peritoneal Cavity during CO_2 Insufflation.

Introduction

There is controversy about the degree of dispersion of cells during laparoscopic procedures. It is not clear from the studies so far, if insufflated gas causes a physical redistribution of cells within the peritoneal cavity at the time of the surgical procedure (Hubens *et al,* 1996). If redistribution of cells occurs, then cells may be able to implant and grow, provided the conditions for growth in the peritoneal cavity and wounds are favourable.

Aim of the study

This study was performed to enumerate the cell distribution immediately following open surgery or laparoscopy. A further aim of this study, was to ascertain if laparoscopy with CO_2 results in a different distribution of cells within the peritoneal cavity, when compared to the gasless and open technique.

Materials and Methods

Twenty four rats were used for these studies. Five rats underwent laparoscopy with conventional CO_2 pneumoperitoneum. Thirteen underwent gasless laparoscopy and six underwent laparotomy. All procedures were performed under anaesthesia and sterile conditions. The laparoscopic and open procedures were performed in the standardised fashion as described earlier.

A suspension of 2 x 10^8 DAMA cells in 2.0 ml of sterile PBS was prepared using the methods described earlier. These cells were then radio-labelled with ^{51}Cr-sodium chromate as described in the earlier section on the methodology for radio-labelling (Chapter 8.1) (Iverson, 1973). The radio-labelled DAMA cells (approximately 1 x 10^8) in 0.4 ml suspension, pre-measured for radioactivity, were introduced into the left upper quadrant of the animal's peritoneal cavity. The initial activity of the cell suspension was measured before the intraperitoneal injection and then the residual activity in the syringe was measured after the injection, from this the total injected dose into the peritoneum was calculated. The rats then underwent laparoscopy with/ without CO_2 insufflation or laparotomy. Thirty minutes after the introduction of the radio-labelled DAMA cells, the rats were killed. The radioactivity present on the peritoneal surface of the abdominal wall was determined from samples using a gamma counter and expressed as a percentage of the intraperitoneally injected dose and thus the quantity of cells (Iverson, 1973).

Laparotomy

Rats underwent a conventional laparotomy through a 3 cm long midline abdominal incision. The radio-labelled tumour cell suspension was introduced slowly into the left upper quadrant of the peritoneal cavity through syringe. Care was taken not to inadvertently contaminate the abdominal wound or any other site with the suspension. The abdominal cavity was then left open for 30 minutes then closed with sutures.

Laparoscopy using carbon dioxide pneumoperitoneum

Rats underwent laparoscopy facilitated by conventional CO_2 pneumoperitoneum. Pneumoperitoneum was initially established using the standard method as described earlier (Chapter 2). A 20 gauge intravenous cannula was introduced through the left upper quadrant of the abdominal wall. The radio-labelled tumour suspension was introduced slowly through this cannula, before sealing the cannula to prevent gas leakage and back flow of injected tumour cells. An 18 gauge

intravenous cannula was introduced into the abdominal cavity through the left lower quadrant abdominal wall and left 'open' to air. This acted as a venting cannula, ensuring a constant circulation of carbon dioxide through the peritoneal cavity. Pneumoperitoneum was maintained for 30 minutes and all ports were left in place until the abdominal wall was harvested for sampling. The ports were removed just before a sample of tissue was taken, to include the site where the ports were situated.

Laparoscopy using a gasless technique

In this group, rats underwent laparoscopic surgery using an identical protocol to the conventional laparoscopy group, except CO_2 insufflation was omitted and a gasless technique was used. Similar to the laparoscopy with CO_2 group, a 20 gauge and an 18 gauge intravenous cannula were introduced separately into the left upper quadrant and the left lower quadrant respectively and the radio-labelled tumour cell suspension was slowly introduced into the peritoneal cavity through the left upper quadrant cannula. This cannula was then closed to air and the left lower quadrant port was left open to atmospheric air. All ports were left in place for 30 minutes.

The animals tolerated the surgical procedures well and were killed at the end of the procedure by intra-cardiac injection of KCl and the abdominal wall tissue was sampled by the method described below.

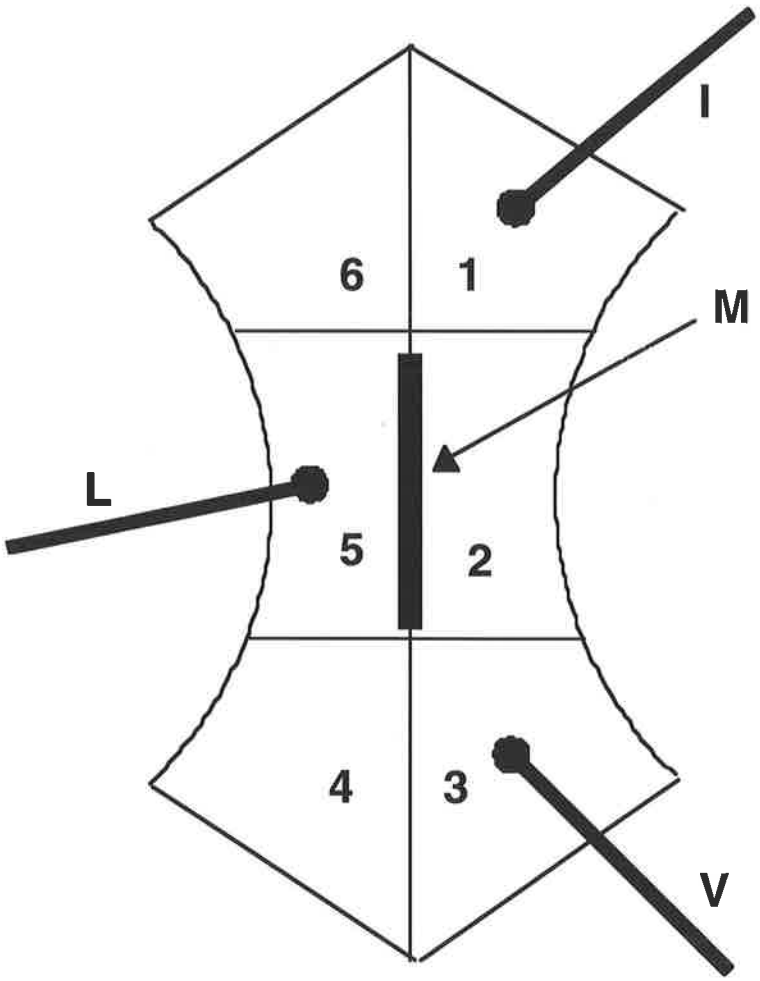
Sampling of the abdominal wall

The abdomen was opened, taking care not to cause movement of the abdominal wall and disturb the intra-abdominal contents. The abdominal cavity was divided into six sectors (Figure 9.1) and samples of abdominal wall were taken using a standard punch (0.5 cm diameter) from the anterior and lateral abdominal wall, for each sector. Representative samples were also taken from the port sites. These tissue samples were counted in a gamma counter (Compugamma, LKB Wallace) and the amount of radioactivity in each of the tissue samples measured. The results were calculated as a

percentage of the total 'initial dose' injected into the peritoneal cavity. The amount of radioactivity represented the number of cells which had adhered to the abdominal wall.

FIGURE 9.1

ABDOMINAL CAVITY DIVIDED INTO SIX SECTORS



I - Injection cannula

V - Venting cannula

L - Laparoscopic cannula

M - Midline incision

Statistics

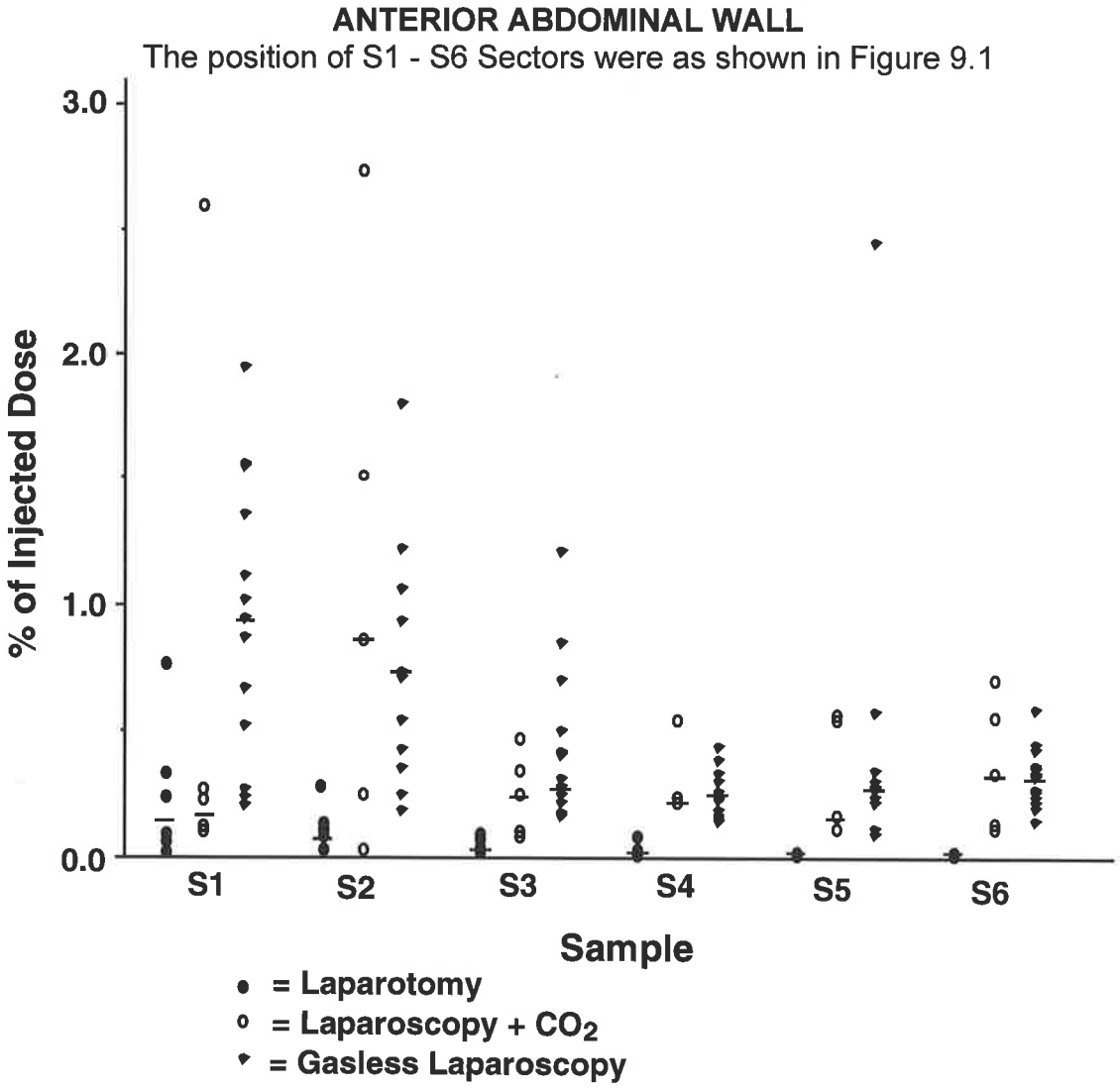
Data was analysed using the medians of the values obtained from the samples for each sector and each group. A Kruskal-Wallis Nonparametric ANOVA test was performed to evaluate the differences between the 3 groups, with a Dunn's multiple comparison test when the Kruskal-Wallis test was statistically significant.

Results

In the laparotomy group, when the abdominal cavity was opened for sampling, it was observed in all the animals, that there was a considerable pool of the cell suspension in the left upper sector, where the cell suspension had been introduced; and along the left paracolic gutter, from where the cell suspension ran out of the rats' abdominal cavity. This observation was less noticeable for the gasless group and very little cell pooling was observed for the laparoscopy with gas insufflation group.

The percentage of injected dose for each of the sector sampling sites (S1 - S6) from the anterior (A) and lateral (L) abdominal walls are shown in Figure 9.2 and Figure 9.3.

FIGURE 9.2

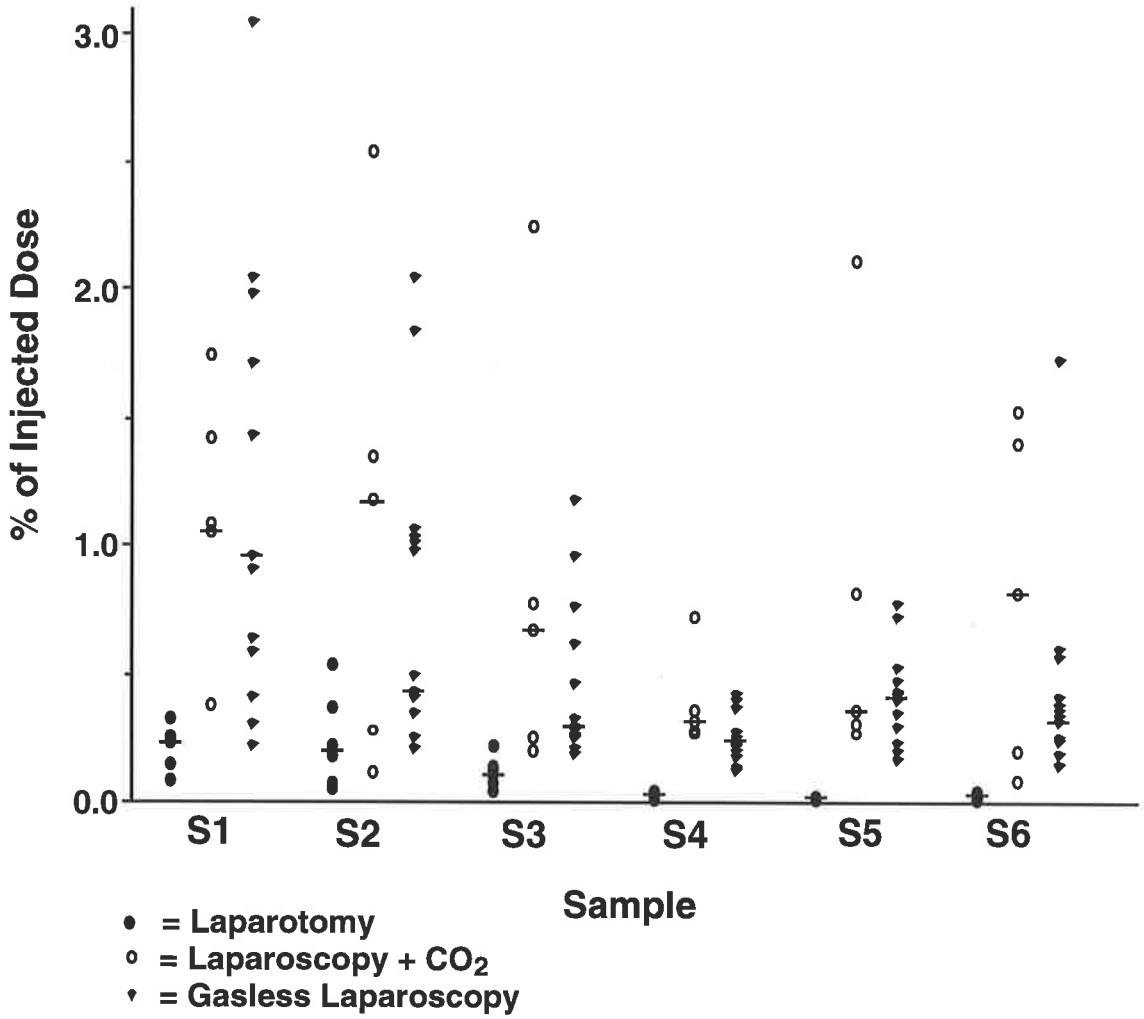


There was less contamination of the sampling sites on the anterior abdominal wall in the laparotomy group and there was no difference between the gas and gasless laparoscopy groups.

FIGURE 9.3

LATERAL ABDOMINAL WALL

The position of S1 - S6 Sectors were as shown in Figure 9.1



The dispersion of cells on the lateral abdominal wall was greater in the laparoscopy group when compared to the laparotomy group.

TABLE 9.1

ANTERIOR ABDOMINAL WALL (SA)

	Column 1	Column 2	Column 3	P value		
Sectors	**Laparotomy**	**Laparoscopy + CO_2**	**Gasless Laparoscopy**	**Column 1 *vs* 2**	**Column 1 *vs* 3**	**Column 2 *vs* 3**
S1A	0.18	0.27	0.96	N S	< 0.05	N S
S2A	0.11	0.87	0.72	N S	< 0.05	N S
S3A	0.06	0.25	0.31	N S	< 0.001	N S
S4A	0.02	0.24	0.26	< 0. 05	< 0.01	N S
S5A	0.01	0.17	0.24	< 0.05	< 0.01	N S
S6A	0.02	0.34	0.32	< 0.05	< 0. 01	N S

On the anterior abdominal wall there was an increase in the dispersion of cells in the laparoscopic group when compared to the laparotomy group.

TABLE 9.2

LATERAL ABDOMINAL WALL (SL)

	Column 1	Column 2	Column 3	P value		
Sectors	**Laparotomy**	**Laparoscopy + CO_2**	**Gasless Laparoscopy**	**Column 1 *vs* 2**	**Column 1 *vs* 3**	**Column 2 *vs* 3**
S1L	0.23	1.08	0.94	< 0.05	< 0.01	N S
S2L	0.20	1.18	0.49	N S	N S	N S
S3L	0.11	0.67	0.33	0.05	< 0.01	N S
S4L	0.02	0.32	0.23	< 0.001	< 0.01	N S
S5L	0.30	0.36	0.41	N S	N S	N S
S6L	0.02	0.82	0.34	< 0.01	0.01	N S

On the lateral abdominal wall there was an increase in the dispersion of cells in the laparoscopy group in all sectors, except sectors 2 and 5, when compared to the laparotomy group.

TABLE 9.3

PORT SITES

PORT SITES		Laparoscopy + CO_2	Gasless Laparoscopy	P Value
Injection port	Median	0.2	1.12	< 0.05
	Confidence interval	0.2, 0.46	2.1, 5.95	
Laparoscopy port	Median	0.44	0.22	> 0.05 NS
	Confidence interval	0.15, 1.33	0.13, 1.16	
Venting port	Median	0.08	0.4	> 0.05 NS
	Confidence interval	0.02, 0.86	0.23, 1.12	

When the data from the port sites were compared there was no difference between the port sites, except for the port through which the cells were injected.

DISCUSSION

Ports and incisions are situated on the anterior and lateral aspect of the abdominal wall in the majority of patients undergoing laparoscopic surgery in the clinical situation. So the area of interest of this study was the distribution of cells on the anterior (SA) and lateral abdominal wall (SL), and only sampling of the anterior and lateral abdominal wall was performed.

The findings from this study indicate that free cells undergo a greater dispersion within the abdominal cavity in laparoscopic procedures than in the open procedure. However, the distribution of cells within the laparoscopic groups was similar in both the CO_2 insufflation group and the gasless laparoscopy group.

On the anterior abdominal wall (SA), the dispersion of cells was significantly increased in the sectors opposite the site of injection in the laparoscopy groups compared to the laparotomy group. There was no difference in the cellular dispersion in the sectors of the abdomen on the same side as the injection port, between any of the three groups. On the lateral abdominal wall (SL), there was a significant increase in the dispersion of cells to all sectors in the laparoscopy groups, compared to the laparotomy group except sectors 2 (SL2) and 5 (SL5). These were the two lateral sectors in which the ports were maintained (the venting port in sector 2 and the laparoscopic port in sector 5). However, there was no significant difference in the dispersion of cells on the anterior and lateral abdominal walls between the groups that underwent laparoscopy with gas and those that underwent laparoscopy without gas.

The total amount of radioactivity on the abdominal wall in the laparotomy group was less compared to the laparoscopy groups and this is most probably because the cells pooled in one place and thus there was very little distribution of cells to the rest of the abdominal cavity. This was confirmed by the observation that in the laparotomy group at the conclusion of the experiment, there was an appreciable amount of liquid in the vicinity of where the cell suspension was instilled. This was less in the gasless and

almost not detectable in the CO_2 laparoscopy group. This was a very subjective observation, as there was no sampling to quantify this pool of cell suspension or the amount of cell spread to the bowel and other organs within the abdomen. However, this observation could also be explained by the evaporation of the liquid pool, by the flow of gas over its surface.

There was no significant difference in the number of cells found in port sites in laparoscopy with gas group and the gasless laparoscopy group. In this study, all three cannulae were left in place till the harvest of the abdominal wall specimens. The cannulae were removed just before sampling and the sites where the cannulae were situated were sampled. The possibility of cannulae being contaminated during the procedures and hence the spread of tumour cells to the abdominal wall and port sites, cannot be entirely ruled out. It is known that cells have an affinity for injured or raw surfaces. Thus cells may be deposited in raw areas like wounds irrespective of the procedure. Even though there was no difference in cell deposition between the two laparoscopy groups, subsequent implantation and growth could be eventually different.

The results need interpretation in the light of the methodology used in this study. The experiments were not randomised and therefore inter-group variations of cell kinetics and behaviour cannot be ruled out, although this is unlikely. The amount of cell adherence to the posterior abdominal wall and peritoneum was not measured. It was assumed that the remaining cells, apart from those which were found on the harvested abdominal wall, remained in the abdominal cavity. From the data collected in this study, it is not possible to determine the distribution pattern in the rest of the abdominal cavity.

In the gasless laparoscopy group, the abdominal wall was suspended with sutures to create a working space, but this was not a rigid arrangement and the ports were left open to the atmosphere. It is possible that the respiratory movement of the abdominal wall may have produced a flow in and out of the peritoneal cavity. In this

group this may have contributed to or caused the distribution of cells within the peritoneal cavity. This could also occur in clinical settings.

The cannulae remained *in situ* until the abdominal wall was sampled, the cannulae were then removed and the abdominal wall sampled to include the site of the wound through which the cannulae were introduced. It is possible that the cannulae came into contact with the abdominal contents and radio-labelled cells, and this in turn caused more deposition of cells in the port sites and around the abdominal wall site where the cannulae were situated. This would explain the increased distribution of cells on the lateral abdominal wall in the sectors through which the ports were placed (SL2 & SL5).

These factors may have influenced the findings which show that the cells are distributed within the peritoneal cavity to the same extent after gasless laparoscopy, as that in laparoscopy with CO_2 insufflation. These results seem contrary to the findings of the study described earlier in this thesis, where there was evidence of a significant amount of cells being transported through the venting gas following laparoscopy with CO_2 insufflation and not in the gasless laparoscopy group. However, the earlier study did not address the issue of dispersion of cells within the peritoneal cavity. The earlier study only addressed the issue of the ability of high flow gas leaks to transport free cells.

The venting gas study and the present study differ in the tumour dose and gas flows used. The cell content and volume of tumour cells introduced into the peritoneal cavity was much higher in the venting study (Chapter 8.2), as were the gas flow rates. Factors such as the rate and pressure of gas flow may also influence the degree of cell distribution (Jones *et al,* 1995; Knolmayer *et al,* 1996).

Alternatively, this may be a genuine effect which would indicate that free cells in the peritoneal cavity may spread within the peritoneal cavity by capillary action, or

by direct contact with bowel loops and movement of the abdominal wall against bowel loops. This could happen irrespective of the presence or absence of circulating gas, as long as a dead space within the peritoneal cavity was present and movement of air or gas in and out occurred either passively or actively. The differences seen in the laparotomy and laparoscopy groups may be, that in laparoscopy the abdominal cavity is an enclosed space (where factors such as movement of air or the presence of instruments may be important) compared to laparotomy, which is an open cavity.

The normal peritoneum is resistant to the implantation of free tumour cells and their growth (Jones & Rous, 1914; Weiss, 1996). While this study has shown a greater dispersion of tumour cells in both the laparoscopy groups, it is only indicative of the potential of tumour cells to implant. This study does not address the relationship between the ability of tumour cells to implant and subsequently grow, factors such as: the behaviour of shed tumour cells and the metastatic potential of cells from tumours of different tissue origin, may determine successful implantation and growth (Tanida *et al,* 1982; Fermor *et al,* 1986; Miyazaki, 1986; Murthy *et al,* 1991; Pasieka & Thompson, 1992; Asao *et al,* 1994; Yonemura *et al,* 1996). It is known that peritoneal immunity and peritoneal integrity needs to be compromised for tumour cells to implant and grow (Miyazaki, 1986; Yashiro *et al,* 1996; Yonemura *et al,* 1996). It is possible that the circulating CO_2 produces metabolic and immunologic changes in the peritoneal cavity (Volz *et al,* 1996), that may predispose to the enhanced implantation and growth of tumour cells (Volz *et al,* 1996) and in the absence of CO_2, the undamaged peritoneal immune and structural factors are able to destroy or prevent the implantation and growth of tumour cells. This remains unsubstantiated, but if it is one of the mechanisms by which CO_2 causes an adverse effect, then it is logical to explore the effects of chemically inert gases, like helium, on this phenomena.

These findings are in agreement with the findings of another group of investigators who have studied this problem with different free cell models. Allardyce *et al* used HeLa cells labelled with ^{51}Cr isotope and investigated the circulation of a

predetermined quantity of cells within the peritoneal cavity of pigs, following laparoscopy and gasless laparoscopy (Allardyce *et al,* 1996). Their findings indicate that leakage of gas during laparoscopic insufflation is a major cause for tumour cell distribution to port sites. They also concluded from their study that in the gasless series, the contamination of ports was the main mechanism of transport of free tumour cells to port site wounds.

Thus this study indicates that there may be other effects, apart from the physical movement produced by the circulating CO_2, which may explain an increased implantation and growth of tumour in the presence of circulating CO_2 in the peritoneal cavity.

Further studies are indicated to investigate the peritoneal metabolic and immunological changes caused by CO_2. It is also necessary to study the effect of inert gases on the development of this problem.

CHAPTER 10 IMMUNOLOGICAL STUDIES

10.1. Immunological Changes caused by Open Surgery and Laparoscopy.

Background

There is a growing concern that CO_2 used in laparoscopic procedures may have an adverse impact on the prevention of growth and dissemination of tumour cells within the peritoneal cavity (Nduka *et al,* 1994; Savalgi & Rosin, 1996). This attitude of caution is a result of an increasing number of reports of metastases in port site wounds and intraperitoneal spread of tumour cells, following laparoscopic surgery for malignant disease (Martinez *et al,* 1995; Johnstone *et al,* 1996). There is controversy as to the pathophysiology of this phenomenon. It is unknown if this effect is due to the physical effect of gas distributing the cells around the peritoneal cavity (Jones *et al,* 1995; Allardyce *et al,* 1996), or if changes in tissue metabolism occur. It is also possible that alteration in the immune status within the peritoneal cavity or wound that occurs, may be responsible for metastatic growth (Rae *et al,* 1995; Volz *et al,* 1996). The clinical and laboratory studies so far have not been able to clarify this issue.

Peritoneum not only forms a mechanical cover with a smooth surface for the internal organs, but it also contains a multi-layered and complex defence system that protects it's serosal surface (Tsai *et al,* 1995; Heel & Hall, 1996). It is now becoming evident that the exposure of the peritoneum to antigens can result in a specific lymphocyte mediated immune response (Pancorbo *et al,* 1994; Heel & Hall, 1996).

It is generally accepted that laparoscopic surgery causes less immuno-suppression in the post operative period, compared to open surgery (Watson *et al,* 1995b; Karayiannakis *et al,* 1997). It has also been postulated that laparoscopic surgery

causes decreased physiological stress, compared to open surgery. However this has not been substantiated. It is known that physiological stress and trauma can alter immune function and more specifically, macrophage function (Wang *et al,* 1980). There is evidence to suggest that the immune response following abdominal surgery may originate in the peritoneal environment (Little *et al,* 1993; Watson *et al,* 1995b). The macrophages resident in the peritoneal cavity form an essential component of this response (Heel & Hall, 1996). The alteration in macrophage function in response to open or laparoscopic surgery, may indicate the level of physiological stress and alteration in immunity of the animal (Iwanaka *et al,* 1997). This may have an influence on the spread of tumour within the peritoneal cavity (Trokel *et al,* 1994).

It has been demonstrated that activated peritoneal macrophages produce Tumour Necrosis Factor - alpha (TNF-α) which is known to have anti-tumour activity (Nilsson *et al,* 1996). It has been demonstrated that peritoneal macrophages from tumour bearing rats have altered activity (Stovroff *et al,* 1989). Therefore, the tumour bearing state has an immune modulating effect in its own right.

10.2. Altered Peritoneal Environment: A Possible Explanation for Port Site Metastases - Indirect Evidence

Introduction

Post operative immune function and cell mediated immunity are known to be significant denominators for patient outcome following surgical procedures (Karayiannakis *et al,* 1997). Macrophages form an important component of the peritoneal immune system. It is possible that an explanation for port site metastases may be related to an alteration in the peritoneal environment and cellular immunity, brought about by the CO_2 pneumoperitoneum. Studies have demonstrated that the presence of a primary tumour and the introduction of non-specific antigens, such as Bacterial endotoxin (Lipopolysaccharide *Escherichia coli* 0127 BS) can cause significant alteration in the activation of peritoneal macrophages and possibly other cells (Stovroff *et al,* 1989). In a study by Watson *et al,* 1995b, comparing the peritoneal macrophage activation following laparoscopy with air or carbon dioxide and laparotomy, they found that there was decreased priming or activation of the peritoneal macrophages in the CO_2 insufflation group. This was detected by the decreased production of TNF-α by the peritoneal macrophages following stimulation with lipopolysaccharide (LPS). They concluded that it was factors in the air rather than from the wound that were responsible for the early immunological alterations induced in the host following surgery. It was postulated that factors in the circulating air induced translocation of endotoxin and this in turn acted on the mononuclear phagocytes and neutrophils in the peritoneal cavity and systemic circulation (Watson *et al,* 1995b).

In another study by Iwanaka *et al,* 1997, where the peritoneal macrophage numbers were studied following laparotomy, laparoscopy with CO_2 and gasless CO_2, they found that the laparotomy group had the lowest number of macrophages when compared to the CO_2 group.

In another study by Murphy *et al*, 1988, where they introduced Pristane into the peritoneal cavity prior to intraperitoneal tumour cell injection, they found that the macrophage response was also associated with potentiated tumour growth. They postulated that this potentiation may be due to factors produced by macrophages. The immunological consequences of laparoscopy, with its relatively clean atmosphere and laparotomy with the relatively contaminated atmosphere, may be manifested primarily in the peritoneal cavity and may also be the major site of immune change in the tumour bearing state (Watson *et al,* 1995b).

Aim of the study

In this study, alterations in the immune status and peritoneal environment were induced by primary tumour implantation or an intraperitoneal injection of LPS. The pattern of spread of free tumour cells, in the peritoneal cavity of the rats, was subsequently studied.

Materials and Methods

Experiment - 1

Tumour bearing rats with intraperitoneal injection of free tumour cells

Eighteen rats were used for this study. The rats were implanted with tumour in their abdominal wall 7 days before the procedure. At one week, when all rats had tumours in their abdominal wall, rats underwent an intra peritoneal injection of free tumour cells during open surgery, laparoscopy with CO_2 or gasless laparoscopy. The procedures were performed under general anaesthesia, the surgical approach was as described earlier in this thesis. Free tumour cells were prepared from harvested tumours of carrier rats according to the technique described in Chapter 5. The final concentration was adjusted to give 1×10^7 cells in 200 µl of sterile PBS. This was then introduced into left upper quadrant of the peritoneal cavity of all animals, at the commencement of one of three surgical procedures. The primary tumour was left undisturbed.

In the laparotomy group, the abdominal cavity was kept open for 30 minutes. While in the laparoscopy group, the procedure lasted for 30 minutes with or without gas insufflation. The animals were allowed to recover from the surgery. The animals were maintained in a similar fashion as described earlier. At the end of 6 days, the rats were killed and an autopsy was performed. The pattern and nature of macroscopic spread of the tumour cells was recorded according to the peritoneal cancer index described in Chapter 5.

Experiment - 2

Normal rats with intraperitoneal injection of tumour cells

(no operative procedure performed)

Twelve non tumour bearing rats were used for this part of the study. In six non tumour bearing rats, free tumour cells were injected intra-peritoneally into the left upper quadrant and these acted as controls. In the other six non tumour bearing rats, LPS (400µg *E. Coli* 0111.B4, Sigma) was injected intra-peritoneally 4 hours prior to injecting free cells into the peritoneal cavity. Both groups of rats did not undergo any further surgical manipulation. All procedures were performed under anaesthesia. The rats were allowed to recover and on the sixth day, the rats were killed and an autopsy was conducted. The peritoneal cavity was examined for the pattern of tumour spread, the growth of tumour and graded according to the peritoneal cancer index described earlier.

In both the experimental groups, a large inoculum of tumour cells was used to investigate the potential effect of tumour bearing state and the state of altered peritoneal environment on intraperitoneal spread, growth and implantation and would be comparable to the similar study on non tumour bearing rats performed earlier (Chapter 5). The protocol of intraperitoneal tumour dose, operative procedures and procedure duration was identical to the previous study, with non tumour bearing rats.

Statistics

Data were analysed using Fisher's exact test.

Results

Experimental Model - 1

Tumour bearing with intraperitoneal injection of free tumour cells

In the tumour bearing group, there was a significant degree of spread and growth of tumour on the incision and all the sectors of the peritoneal cavity, irrespective of the operative procedure. When the tumour distribution and growth at the wounds and sectors of the peritoneum were compared to the group of normal rats, which underwent an identical procedure, there was a significant increase in growth in all the groups, but this growth was more pronounced in the laparotomy and gasless laparoscopy groups.

The data from the present study was compared with the data from the study on non tumour bearing rats performed in an earlier study (Chapter 5).

TABLE 10.1

MACROSCOPIC TUMOUR SPREAD

(rats with tumour spread/ total number rats in group)

Laparotomy

	Normal rats (n = 12)	Tumour bearing rats (n = 6)	P value *
Incision	1 / 12	4 / 6	P = 0.0217
No of sectors with Grade 3 Tumour	25 / 72	25 / 36	P = 0.001

Gasless laparoscopy

	Normal rats (n = 12)	Tumour bearing rats (n = 6)	P value *
Laparoscopic port	2 / 12	5 / 6	P = 0.0128
Venting port	5 / 12	4 / 6	NS
No of sectors with Grade 3 Tumour	13 / 72	21 / 36	P = 0.0001

Laparoscopy with CO_2

	Normal rats (n = 12)	Tumour bearing rats (n = 6)	P value *
Laparoscopic port	12 / 12	6 / 6	NS
Venting port	11 / 12	6 / 6	NS
No of sectors with Grade 3 Tumour	38 / 72	35 / 36	P = 0.0001

* = Fisher's exact test

Experimental Model - 2

Non tumour bearing rats with intraperitoneal injection of LPS + Tumour cells

In this model, the tumour growth and implantation was confined to the same side of the peritoneum as the injection in 5 of 6 the animals; and in one animal there was tumour spread to only one sector of the opposite side. In the LPS group, all the animals had tumour spread to all the sectors of the abdomen, including the ones on the opposite side to that of the injection. In this experiment, the normal non LPS injected rats acted as controls.

TABLE 10.2

NUMBER OF SECTORS WITH MACROSCOPIC TUMOUR

(6 rats x 6 sectors)

Normal rats	Rats with LPS	P value *
19 / 36	36 / 36	P = 0.0001

* = Fisher's exact test.

Discussion

The mechanism of peritoneal dissemination and implantation of tumour is not fully understood. However, an understanding of the mechanism of peritoneal implantation and dissemination of tumour cells is of significant importance to the efficacy of laparoscopy in tumour resection. There is evidence to indicate that alteration in the peritoneal environment and the status of peritoneal immunity may play a role. It has also been documented that alterations in post operative immune status may originate in the peritoneal cavity (Redmond *et al,* 1992; Watson *et al,* 1995b; Badia *et al,* 1996). The central issue is whether changes in peritoneal immunity or environment, play a part in the development of tumour metastasis. The presence of CO_2 in the peritoneal cavity is known to cause significant metabolic changes in the peritoneum, including altered pH, altered stress hormone response and alterations in peritoneal immunity (Hunter, 1995; Koivusalo *et al,* 1996; Mikami *et al,* 1996). However, it is not clear if these resultant changes contribute to the development of port site metastases.

In the present study, two scenarios were altered: immune status and peritoneal environment (which can occur as demonstrated by other workers) (Stovroff *et al,* 1989; Nilsson *et al,* 1996) and were examined with regard to the pattern of tumour cell implantation.

In the first scenario of the tumour bearing state, there was a significant increase in the amount of tumour growth in the incisions and all sectors of the peritoneum of the laparotomy and gasless laparoscopy groups, compared to a similar group of non tumour bearing rats studied earlier and reported in Chapter 4. In the CO_2 laparoscopy group, the port site metastases were maximal and there was a significant increase in the spread and growth of tumour within the peritoneal cavity of tumour bearing rats. The differences seen in this study are due to changes in the immune status of the animal, which were specifically induced for this study. This finding indicates that alterations in

the immune status of the tumour bearing hosts can influence the pattern of spread of tumour in the peritoneal cavity.

In the second scenario, the rats injected with LPS had a significantly increased tumour cell distribution and altered pattern of implantation. LPS is known to alter the immune status of the peritoneal environment. The increased spread of tumour within the peritoneal cavity without any surgical intervention, indicates that changes in peritoneal environment alone can facilitate implantation of tumour. Endotoxin produced by the bacteria in the gut is a source of LPS. In a study on pigs, by Volz *et al*, increased circulating levels of endotoxin were detected after laparoscopic procedures (Volz *et al,* 1996).

Carbon dioxide is known to cause acidosis of the peritoneal cavity and the resultant acidity can act as a strong irritant to the peritoneal surface. This irritant effect may produce changes which may contribute to the facilitation of peritoneal spread and wound recurrence of tumour (Volz *et al,* 1996). In a study conducted by Iwanaka *et al* in mice, the level of TNF-α production by LPS stimulated macrophages following CO_2 insufflation was significantly less than in open surgery, indicating that the degree of macrophage activation in the presence of a CO_2 pneumoperitoneum is decreased (Iwanaka *et al,* 1997). The metabolic environment associated with low pH leading to increased lactate production, has been suggested to lead to activation of macrophages producing angiogenic factors, which enhance tumour growth (Stovroff *et al,* 1989).

In summary, this preliminary study indicates that:

a. the tumour bearing state causes increased metastatic spread, regardless of the nature of the operation.

b. LPS induced changes in the peritoneal environment causes increased spread and implantation of tumour.

CHAPTER 11 IMMUNOLOGICAL STUDIES

11.1 Peritoneal Macrophage Activation with Laparoscopic, Gasless and Open Surgery.

Introduction

Surgical trauma produces a broad spectrum of immunological changes in the patient (Lennard *et al,* 1985). The implications of these changes are that host defences may be compromised by surgical procedures, thus providing a fertile soil for bacterial invasion and tumour cell metastases. This immunosupression may predispose to infective complications and seeding of tumour after surgery (Redmond *et al,* 1992; Griffith *et al,* 1995). Studies have confirmed the occurrence of post operative immunosuppression after laparotomy and that violation of the peritoneal environment is a critical factor in the induction of this immuno depression (Little *et al,* 1993). Pro-inflammatory mediators are believed to mediate the host response to surgical trauma. This has been predominantly related to wound factors and anaesthesia (Watson *et al,* 1995b). However, the mechanism by which immune suppression is caused after surgery is not clear.

Laparoscopic surgery has gained wide acceptance and the main advantages of this approach are the decrease in the wound related morbidity, pain and shortened hospitalisation (Peters *et al,* 1993; McMahon *et al,* 1994a; Watson *et al,* 1995a; Ellstrom *et al,* 1996; Fukushima *et al,* 1996). Many of these advantages have been explained by the assumption that there is less surgical trauma inflicted on patients who undergo laparoscopic surgery, than for those who have open surgery. The diminished acute phase response to laparoscopic surgery may be related to a decreased release of specific circulating factors, such as interleukin-6, from a smaller wound surface. This

has not been substantiated (Ellstrom *et al,* 1996; Fukushima *et al,* 1996; Ortega *et al,* 1996).

A study comparing patients undergoing laparoscopic and open hysterectomy showed the inflammatory response as measured by interleukin IL-6 levels were similar, indicating the same degree of response occurs in both approaches (Ellstrom *et al,* 1996). The proposed advantage of laparoscopic surgery may therefore be attributable to factors other than the degree of tissue trauma inflicted. Laparoscopic surgery is thought to be beneficial to the host, because laparotomy is known to inhibit the immunological response of the host to a greater degree than laparoscopic surgery (Griffith *et al,* 1995). In experimental models, this effect has been shown to be beneficial to the host, in that there is a decrease in the growth of the primary tumour in animals undergoing laparoscopic surgery, compared to those undergoing laparotomy as shown in the work described in earlier chapters and from the work of other investigators (Bouvy *et al,* 1997). However, the changes in peritoneal immunity produced by laparoscopic surgery are not fully understood.

There is evidence to indicate that the immune alteration associated with abdominal surgery originates in the peritoneal cavity and is a consequence of peritoneal violation (Redmond *et al,* 1992; Badia *et al,* 1996). The local immune status in the peritoneal cavity may influence the dissemination and growth of intra abdominal tumour. Thus, it is essential to understand the changes in peritoneal environment caused by CO_2 pneumoperitoneum.

Aim of the study

This study was conducted to examine the magnitude of change induced in the peritoneal cellular response between laparoscopy with CO_2 and gasless laparoscopy, contrasted with open laparotomy in the DA rat. This study measured the peritoneal macrophage population and the degree of activation of peritoneal macrophages by measuring TNF-α levels.

Materials and Methods

Animals

Forty male DA rats were used for the experiments. All animals were maintained in animal housing as described earlier. All animals were allowed free access to food and water and studied in the fed state.

Normal rats

Normal rats (n = 18) divided into three groups underwent either laparotomy, laparoscopy with CO_2 or gasless laparoscopy. There were six rats in each group. The technique of anaesthesia and surgery was a standardised technique used throughout, as described in the previous studies. The rats were allowed to recover following the procedures and then peritoneal macrophages were harvested after 24 hours.

Tumour bearing rats

Rats with tumour (n = 18) implanted on the abdominal wall 7 days prior to the operative procedure were used. They were divided into three groups and each underwent a standard operative procedure of laparotomy, laparoscopy with gas or laparoscopy without gas. The technique of macrophage harvesting was similar to the previous study.

Laparoscopy with CO_2

For the rats in this batch, CO_2 was insufflated through the abdominal cavity by a standardised method as used in all the previous studies. A Veress needle was introduced to the right of the umbilicus through a stab incision and a disposable mini laparoscopy cannula introduced it. A 18 gauge intravenous cannula was introduced into the left upper quadrant, to simulate a laparoscopic port and a 16 gauge intravenous cannula was introduced into the left lower quadrant, to act as the venting cannula. CO_2 was insufflated through peritoneum at a pressure of 0.2 L/ min and at a pressure of 4 mmHg. The insufflation was maintained for 30 minutes; the cannulae were removed

and the wounds sutured with 3-0 Prolene sutures, before the rats were allowed to recover from the anaesthesia.

Gasless laparoscopy

In this group, the rats underwent laparoscopic surgery using an identical protocol to the CO_2 laparoscopy group, except the omission of carbon dioxide insufflation and the abdominal wall was suspended with multiple sutures from a wire frame. The wound was closed with 3-0 Prolene sutures. The rats were allowed to recover from anaesthesia after 30 minutes from the start of the procedure.

Laparotomy

The rats underwent a conventional laparotomy through a 3 cm long midline abdominal incision. The abdominal cavity was left open for 30 minutes and then closed with 3-0 Prolene sutures. The animals were allowed to recover and after 24 hours, the resident peritoneal macrophages were harvested from the animals.

Control rats

Four animals were used as control rats, these animals underwent only anaesthesia and then peritoneal macrophages were harvested.

Peritoneal macrophage harvest

A small area of fur and skin was dissected free of the underlying muscle on the anterior abdominal wall and the muscle swabbed with 70% alcohol. Peritoneal lavage was performed using 10 ml of sterile PBS, introduced through a 22 gauge needle into the peritoneal cavity. Taking care not to puncture any intra abdominal organs, the abdomen of the rat was gently agitated to mix fluid in the cavity. After 2 minutes, the abdominal cavity was opened via a small incision and the lavage fluid aspirated using a blunt 18 gauge needle.

Macrophage culture

The lavage fluid obtained was centrifuged for 7 minutes at 500 g, to form a pellet of cells. The supernatant was removed. The cell pellet was then re-suspended in 1 ml RPMI medium (Trace Bioscience Pty. Ltd.) containing antibiotics and 10% foetal calf serum. Cell counts were performed using trypan blue exclusion. The cells were plated in 6 well plate trays and 4 wells were used per rat. The cell suspension was diluted to make up to a final concentration 1 x 10^6 cells per well. All the groups had an initial macrophage viability of greater than 95%, as determined by trypan blue exclusion. Macrophages were then incubated in an atmosphere containing 5 percent carbon dioxide at 37°C for 3 hours to promote adhesion. The cells were washed twice with PBS, to remove any non-adherent cells, leaving a purified population of macrophages attached to the wells (Haynes *et al,* 1991).

TNF-α measurement

Three millilitres of RPMI was added to the washed macrophages. Each rat had 4 wells, of which two wells contained no lipopolysaccharide (LPS; *E. Coli* 0111.B4, Sigma) and two wells which contained LPS (final concentration 2 μg/ ml). The trays were incubated overnight in an incubator at 37°C. Supernatants were recollected, after overnight incubation, from each well. The supernatant was spun to remove cell debris and stored at -70°C until assayed.

The degree of activation of the macrophages was measured by estimating the levels of TNF-α production by the LPS stimulated and non stimulated macrophages in culture. The TNF-α in the supernatants from the cultures collected was measured using a L929 bioassay (Stovroff *et al,* 1989).

L929 bio assay

TNF-α sensitive L929 cells seated in 96 well plates, 8 x 10^4 cells in each well were incubated overnight. 50 μl of 8 x 100 serial dilutions of known concentration

mouse TNF-α was added as a positive control and some wells contained only RPMI as a standard. Negative controls with 50 µl of 6 x 1:4 dilutions of macrophage solutions were added to wells. Trays were incubated overnight, media removed and then washed with 0.9% NaCl (normal saline). Saline was replaced with 50µl of crystal violet stain in each well. Trays were left for 10 min to allow absorption of stain into live cells. The excess stain was rinsed away and the plates left to dry. 33% acetic acid was used to dissolve the cells and release the stain. Trays were then placed in a plate reader to obtain the optical density values of each well (Stovroff *et al,* 1989).

Statistics

Differences between the groups for the numbers of peritoneal macrophages were compared using the analysis of variance (ANOVA) test. The data from the estimation of TNF-α levels was analysed using an ANOVA and Tukey-Kramer multiple comparison test.

Results

The numbers of peritoneal macrophages harvested from DA rats after each type of operation was determined. Because of the possibility that bacterial contamination might alter the macrophage response, the rats underwent no additional operative manipulation.

In the normal rats, one of the rats which underwent laparotomy had bleeding into the peritoneal cavity and the lavage was blood stained, so the results were considered unreliable and not included in the final analysis

TABLE 11.1

NORMAL RATS

(Macrophages x 10^6, median)

	Control	Laparotomy	Laparoscopy + CO_2	Gasless Laparoscopy
Median No. of macrophages	9.6	9.0	11.1	9.15
Confidence interval	5.2, 17.3	8.2, 10.1	8, 15.7	4.1, 15

ANOVA P = 0.60. N.S.

In the tumour bearing rats, one of the animals had a blood stained lavage and this was discarded, as this was considered unreliable and so was not included in the analysis.

TABLE 11.2

TUMOUR BEARING RATS

Number of macrophages (x 10^6)

	Control	Laparotomy	Laparoscopy + CO_2	Gasless Laparoscopy
Median No. of macrophages	9.6	8.1	8.2	10.2
Confidence interval	5.2, 17.3	7.6, 9.3	5.1, 14.8	7.5, 11.7

ANOVA P = 0.62. N.S.

TABLE 11.3

TNF-α LEVELS

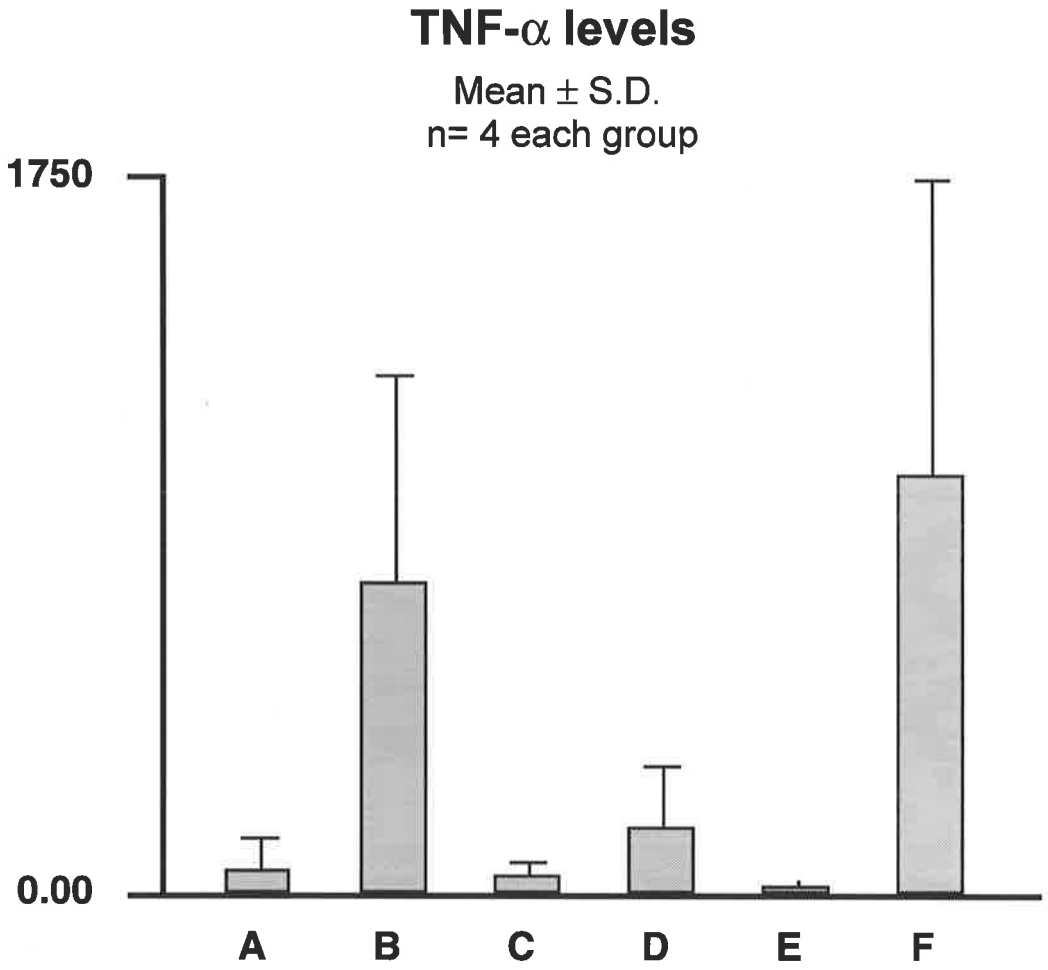
Biologically active units/ μL

Group	Laparotomy		Laparoscopy		Gasless Laparoscopy	
	- LPS	**+ LPS**	**- LPS**	**+ LPS**	**- LPS**	**+ LPS**
Median	13	1024	58	168	14	912
Confidence Interval	4, 22	43, 158	2, 113	50, 443	7, 39	128,2175

ANOVA P = 0.0025.

Tukey-Kramer Multiple Comparisons Test	**P value**
Laparotomy *vs* Gasless Laparoscopy	P > 0.05 N.S.
Laparotomy *vs* Laparoscopy with CO_2	P > 0.05 N.S.
Laparoscopy with CO_2 *vs* Gasless Laparoscopy	P < 0.001

FIGURE 11.1



A	=	Laparotomy
B	=	Laparotomy + LPS
C	=	Laparoscopy with CO_2
D	=	Laparoscopy with CO_2 + LPS
E	=	Gasless Laparosocpy
F	=	Gasless Laparoscopy + LPS

Discussion

In the first part of the present study the difference in macrophage population number was investigated in the tumour bearing and non tumour bearing rats. There was no significant difference in the population of peritoneal macrophages comparing the normal and tumour bearing rats. There was also no significant difference in macrophage numbers between the controls and any of the operated groups, either in the tumour bearing rats or the normal rats. The study in the earlier chapter (10.2) indicated that tumour bearing state adversely affected the pattern of intraperitoneal spread of tumour. The finding from the previous study and the findings of the macrophage population in this study, suggest that this adverse effect in the tumour bearing state as seen in the earlier study may be caused by an altered functional state, or activation of macrophages. This may also be due to changes in other components of peritoneal defence, such as mesothelial cells etc.

In the second part of the study the macrophage activation in the tumour bearing group was investigated, to determine if there was a difference in the state of activation of peritoneal macrophages and whether maximal activation was in the tumour bearing state. At the time of writing, only four of the six rats from each group could be measured for TNF-α levels. Thus the results of this study are preliminary in nature. The TNF-α level of the supernatants was inversely proportional to the dilution of the supernatant necessary to achieve killing of the fibroblast cells. This was expressed as the number of biological units of TNF-α present in the supernatant. The results from this study indicate that in the control rats there were minimal detectable levels of TNF-α produced by the macrophages even after LPS stimulation. LPS stimulated TNF-α levels in the laparotomy group were higher than that of controls, but did not reach statistical significance when compared to the CO_2 laparoscopy group. There was a higher level of TNF-α in the LPS stimulated macrophages from the gasless laparoscopy group. This difference was statistically significant when compared to the laparoscopy group with CO_2.

Peritoneal macrophages have an important role to play in the local immune response of the peritoneal cavity and serve as scavenger cells (Adams & Hamilton, 1984). Peritoneal macrophages initiate the immune response by the production of one or more cytokines. Macrophages secrete a variety of cytokines that may help modulate the activity of several other immune cells. In addition, some of these cytokines, including TNF-α, possess the ability to kill invading bacteria and tumour cells (Carswell *et al,* 1975; Redmond *et al,* 1992).

Levels of macrophage activity are defined by a hierarchical scheme of macrophage function and designated by various terms such as: resident tissue macrophages; elicited macrophages and activated macrophages (Johnston, 1988). The capacity of unstimulated resident macrophages to produce TNF-α in response to endotoxin stimulation is minimal (Stovroff *et al,* 1989).

The increased production of TNF-α following stimulation by endotoxin in studies on tumour bearing rats, suggests an increased or altered level of macrophage activation (Johnston, 1988). Macrophage cytokine production is an indicator of physiological stress. Elevated cytokine production by the macrophages harvested from the peritoneal cavity, may also suggest a greater degree of operative stress (Iwanaka *et al,* 1997).

Cellular or molecular mechanisms by which the tumour bearing state alters macrophage function are unclear. The presence of a tumour appears to affect host macrophages both locally and systematically. Although the peritoneal macrophages are in a compartment separate from direct stimulation by the tumour, a mediator of this heightened macrophage activity may be interferon-gamma (IFN-γ)which plays a role in the host defence against tumour (Stovroff *et al,* 1989). High levels of TNF-α may represent a host defence mechanism against tumour challenge. TNF-α acts as an activating agent for macrophages, not only in tumour cell cytolysis (Philip & Epstein, 1986), but also for the increased production of itself and other biologically active cytokines. In addition, it is a chemo-attractant for the recruitment of additional

activated macrophages (Barbul, 1992). Monocyte differentiation and activation is regulated by TNF-α inducing cellular differentiation along monocyte/ macrophage pathways (Nilsson *et al,* 1996). Therefore, macrophages producing TNF-α in response to LPS administration stimulate the differentiation of mononuclear cells from a precursor population (Michie *et al,* 1988).

There is evidence to suggest that lactic acid may play a role in the activation of tumour infiltrating macrophages (Kern & Norton, 1987). Lactic acid itself can act as a metabolic signal to activate macrophages to release angiogenic factors. An anaerobic acidic environment may also be the signal responsible for initiating a cascade effect, with macrophage infiltration and high TNF-α production. Endogenous host cytokine or cytokines produced by activated macrophages in response to an invasive malignant neoplasm can result in systemic effects in the host (Stovroff *et al,* 1989).

Post operative TNF-α levels are found to correlate with wound healing. The effect of a wound alone on early postoperative immune function was eliminated in a study done by Watson *et al*, 1995b, who compared laparotomy with laparoscopy in a murine model. They observed that immune function after both these procedures was similar to that seen in the controls, 24 h post operatively. Watson *et al* have shown that the macrophage activation and TNF-α production is lowest following CO_2 laparoscopy and maximal following laparotomy (Watson *et al,* 1995b). He also postulated that air contamination of the peritoneal cavity, triggers the systemic responses seen through the medium of endotoxin translocation from the gut. Eggermont *et al* (1988) demonstrated that surgery outside the abdomen did not enhance intraperitoneal tumour growth. This suggests that local factors in the peritoneum, may have a more important role to play in the development of immunity against peritoneal dissemination (Iwanaka *et al,* 1997).

Several authors have reported increases in plasma concentration of IL-6, with low or undetectable plasma levels of TNF-α and IL-1, after surgical operations (Badia *et al,* 1996). There are few reports however, on the cytokine response of the peritoneum. A study investigating the cytokine response in the peritoneum following

surgery suggests that the post operative peritoneal response may originate largely from the peritoneal cavity (Badia *et al,* 1996).

Volz *et al*, 1996, observed that there was a reduction in the oxidative burst of granulocytes, following CO_2, therefore it is assumed that there is a profound disturbance of peritoneal conditions during CO_2 insufflation.

Most of the cancer cells entering the metastatic process are killed. Although much emphasis has been placed on the rapid intravascular death of cancer cells during haematogenous metastases, much less is known about the inefficiency associated with peritoneal carcinomatosis (Weiss, 1996). Studies on humans and laboratory animals indicate that most of the cancer cells released into the peritoneal cavity are killed (Jones & Rous, 1914). Some of the mechanisms involved in killing are achieved through cellular defence mechanisms operating in the peritoneal cavity.

The peritoneal defence mechanism also consists of other components such as the mesothelial covering of peritoneum. The mesothelial cell monolayer has been reported to prevent infiltration of cancer cells into the peritoneum (Jones & Rous, 1914). It has been reported that peritoneal fibrosis induced by gastric cancer cells prior to metastasis, may provide a congenial environment for peritoneal metastases (Yashiro *et al,* 1996). Mesothelial cells have been reported to become hemispherical and exfoliate from the peritoneum. Morphological changes in mesothelial cells may be induced by cancer cells or other substances, such as factors produced by peritoneal fibroblasts. Whatever the stimulus, the alteration in the morphology of mesothelial cells results in an environment compatible with peritoneal dissemination of cancer cells (Yashiro *et al,* 1996).

Post operative cytokine response levels may be dependent on the release of specific circulating factors such as cytokine IL-6 from the wound surface. Corresponding changes in the levels of IL-1 and TNF-α may also occur (Mealy *et al,* 1992). However, in this study the increase in TNF-α levels in the gasless laparoscopy

group when compared to the CO_2 laparoscopy group, cannot be explained by the size of the wound, as both the groups of animals had identical wounds.

These preliminary findings raise the possibility that laparoscopy with CO_2 causes a decreased production of TNF-α by macrophages. This difference between the two laparoscopic groups may be caused by the circulating CO_2. It has been demonstrated that circulating CO_2 causes acidosis on the peritoneal surface (Volz *et al,* 1996). Acidic pH of the peritoneal environment is known to cause alterations in the peritoneal defence functions. Thus although laparoscopy results in a better preservation of general immunity, the lowered peritoneal macrophage activation may depress peritoneal immunity. This could be one of the contributing factors in the intraperitoneal dissemination of tumour cells, as one of the functions of TNF-α, produced by activated macrophages, is to kill tumour cells. Macrophages also produce other cytokines such as IL-6, and IL-1 and the variation in the production of these may also influence the peritoneal immune response.

The peritoneum is more than just a mechanical covering that allows organs to glide over one another, it also contains a complex defence system and relies mainly on innate immune mechanisms, such as: anti-bacterial activity of the peritoneal fluid, bacterial absorption, phagocytosis and natural killer cell activity (Heel & Hall, 1996). The intraperitoneal cell mediated immune function and systemic response are likely to play key roles in the patient outcome after abdominal surgery (Badia *et al,* 1996). Studies investigating specific pathophysiological effects of laparoscopy on the peritoneal immune and metabolic environment, may lead to a better understanding of the possible aetiological factors involved in peritoneal spread and port site dissemination of tumour cells. Further studies are clearly warranted to extend these preliminary findings.

CHAPTER 12 IMMUNOLOGICAL STUDIES

12.1. Immune Response in the Incision Wounds following Laparotomy and Laparoscopic Surgery.

Background

Although the concept of seeding of cancer cells in surgical wounds is old, there is controversy regarding its increased incidence following laparoscopic surgery. An increasing number of cases are being cited in the literature, in which the wound has been the site of metastatic focus following laparoscopic surgery for malignant disease (Martinez *et al,* 1995; Johnstone *et al,* 1996). This has drawn attention to possible aetiological factors in the wound itself. Many authors have postulated that the mere presence of a wound can give rise to this phenomenon (Alexander & Altemeier, 1963; Murthy *et al,* 1989). It is known that any injured tissue or site of recent trauma, can be a site for the growth of malignant cells.

It is known that tumour preferentially spreads to recently traumatised tissues (Murthy *et al,* 1991) and that malignant cells grow more easily in areas of high cellular proliferation. The intact peritoneum is resistant to tumour cell implantation (Jones & Rous, 1914), but the port site provides a localised breach of that defence and an area of high cellular proliferation associated with the healing process. The local release of inflammatory regulators associated with this, may provide an environment that facilitates the implantation and subsequent development of tumour metastasis at this site.

Although the "wound factor" does not provide an explanation for all the observed increases in metastases associated with laparoscopic surgery, it is possible that small wounds, such as those created by trocars, have a different mechanism of healing

than large laparotomy wounds. If the immunologic or inflammatory responses of these two different classes of wounds were different, then this could be one of the contributing factors for the development of port site metastases (Rae *et al,* 1995).

There is evidence to indicate that abdominal wall perfusion is impaired during CO_2 pneumoperitoneum (Schilling *et al,* 1997). It has also been demonstrated that vasoactive substances, like vasopressin, are stimulated during laparoscopy and this may compound the problem (Volz *et al,* 1996). An impairment of the abdominal wall perfusion during CO_2 pneumoperitoneum might be an additional factor for the development of implantation metastases in the peritoneum and abdominal wall wounds.

Aim of the study

This study was conducted to investigate if the recruitment of immune cells in the wounds in laparoscopic surgery is different from that in the wounds made during open surgery.

Materials and Methods

Eight normal rats were used for this study. These rats underwent CO_2 laparoscopy or laparotomy. There were four rats in each of the groups. After the procedure, the incisions were closed in the standard fashion used in all the previous studies. A standard suture of 3-0 Prolene was used for closure of all the wounds. The rats were allowed to recover and after 24 hours the rats were killed. All the wounds were excised for immunohistochemistry.

Tissue sampling and preservation

In all animals the wounds were excised fresh from the animals. Five to seven millimetre blocks of tissue were immersed in water soluble mountant OCT 4583 compound (Tissue Tek, Miles Laboratories) in aluminium foil trays. These trays were formed by shaping aluminium foil over a small cylindrical tube. The aluminium foil trays were placed into isopentane slurry close to -180°C. This slurry was formed by

immersion of a 10 ml beaker of isopentane into a container of liquid nitrogen at -180°C. This method provided a thermal buffer for an even rapid freezing of the tissue. The tissue was well preserved for immunohistology. The specimens were stored in -70°C till tissue sectioning.

Tissue sectioning

Frozen tissue blocks were cut at 4 μm serially (Leica CM 1500 Cryostat). These sections were mounted on acid-cleaned gelatinised slides, fixed with acetone for 10 minutes and air dried at room temperature.

Immunohistochemical techniques

All monoclonal antibodies used were anti- rat antibodies diluted in 10 % normal rat serum (Dijkstra *et al,* 1994). For immunohistochemical staining, all reactions were carried out at room temperature. Sections were rinsed three times in PBS between each staining step. After blocking with 10 % normal rat serum for 30 minutes, the sections were incubated overnight with the primary antibody, at previously determined concentrations in a humidified box. The sections were then biotinylated with a second antibody for 60 minutes. The endogenous peroxidase was then blocked (methanol and 0.5% H_2O_2 x 20 minutes). This was followed by incubation in horse radish peroxidase (HRP) for 60 minutes. The sections were then allowed to react with nickel chloride, enhanced with diaminobenzidene (DAB) and counter-stained with methyl green. The slides were rinsed in water, before dehydrating with absolute alcohol. The slides were then mounted. Sections from the rats' spleens were used for positive controls. Sections were treated as outlined above and also with 10 % normal rat serum instead of the first antibody, to serve as the negative controls (Coventry *et al,* 1994; Coventry *et al,* 1995).

Antibody details

TABLE 12.1

ANTIBODY USED IN THE EXPERIMENT

Antibody	Cellular expression	Isotope	Concentration
ED2	resident macrophages	IgG 1	1 : 1000

An initial histochemical study of the wounds revealed that the predominant infiltrate in the wound was macrophages and hence only the assessment of macrophage population was performed using the ED2 marker, which is specific for mature macrophages (Dijkstra *et al,* 1994).

Quantification of positive cells

The density assessment was calculated by counting 50 random sequential fields per frozen section (field size 0.375 mm; diameter 0.44 mm, 400 X magnification).

Statistics

The data from the various groups were compared using the analysis of variance (ANOVA) test and significant differences between groups was assessed by the Tukey-Kramer multiple comparison test.

Results

The findings from this study show that there is a significant smaller macrophage population in the laparoscopic wounds, when compared to the wounds made for open surgery. All the operative interventions were comparable as to the anaesthetic and duration of procedure, the main difference being the size of the incision.

TABLE 12.2

MACROPHAGE COUNTS

(x 25 HP fields, medians)

Site of sample	Median	Confidence interval
OPEN WOUND 1	16.5	15.1, 19.6
OPEN WOUND 2	15.0	12.3, 17.7
OPEN WOUND 3	15.0	12.6, 17.0
OPEN WOUND 4	17.0	14.3, 19.3
GAS LAPAROSCOPY 1	3.0	2.1 , 3.7
GAS LAPAROSCOPY 2	2.0	1.7, 2.4
GAS LAPAROSCOPY 3	2.0	1.3 , 2.2
GAS LAPAROSCOPY 4	2.0	1.7, 2.9
GAS VENTING 1	2.0	1.6, 2.5
GAS VENTING 2	1.0	0.8, 1.6
GAS VENTING 3	1.0	0.9, 2.1
GAS VENTING 4	2.0	1.1, 1.9

Tukey-Kramer Multiple Comparisons Test	P value
Open Wound *vs* laparoscopic port site wound	$P < 0.001$
Open Wound *vs* venting port site wound	$P < 0.001$
Laparoscope port wound *vs* vent port site wound	$P > 0.05$ N.S.

Discussion

The findings from this study show that the laparoscopic wound has significantly less macrophage infiltration when compared to the laparotomy incision. This could indicate that the laparoscopic wound is different in the pattern of infiltration of immune cells compared to that of the laparotomy wound. It is possible that the laparoscopic wound by virtue of its size produces a decreased immune response with a reduction in infiltration of immune cells of which macrophages are a major constituent. Tissue macrophages apart from playing a role in healing also initiate cell-mediated response to pathogens and malignant cells. Thus it is logical to postulate that the laparoscopic wound by virtue of its attenuated macrophage response, is more prone to tumour cell implantation than the laparotomy wound.

There has been considerable interest and debate about the influence of immune and metabolic status of the host on healing wounds (Levenson & Demetriou, 1992) and the influence of the wounds on host immunity (Eggermont *et al,* 1988; Lazarou *et al,* 1989), though the extent of this association is not fully understood (Murphy *et al,* 1988). It has been postulated that the wound elicited immunosuppressive factor is contained within the wound and plays a role in the regulation of wound healing. Fluid from healing wounds has been found to impair *in vitro* lymphocyte response to allogenic stimulation (Eggermont *et al,* 1988). A study on mice demonstrated that small inocula of liposarcoma cells, which do not give rise to tumours when inoculated into normal skin, lead to tumour formation when inoculated into wounds (Baker *et al,* 1989). It has been demonstrated that tumour growth is enhanced and accelerated by healing tissues during the early stages of healing. However, the nature of the factors influencing tumour growth, expressed by the healing wound is not fully known (Weese *et al,* 1986).

The formation of local recurrence of tumour is also poorly understood. Inadvertent spillage of cancer cells into the operative field or direct contamination of the wound, have been suggested to explain wound recurrence (Fortner, 1960). It has

been demonstrated that exfoliated cells can proliferate and metastasise (Fermor *et al,* 1986). The propensity for seeding of transitional cell tumours to wounds, has been well known for many years (Andersen & Steven, 1995). It has been demonstrated by cell dilution assays that fewer tumour cells are required to induce tumour growth in skin incisions, than in intact skin (Baker *et al,* 1989). This has been challenged by Arons, based on a study that showed no correlation between the presence of tumour cells in the wound washings and the frequency of local recurrence (Arons *et al,* 1961). Laboratory studies to investigate the influence of surgical trauma on the promotion of metastases and the mechanisms that facilitate tumour implantation or growth, have so far been inconclusive.

Murthy *et al,* 1989, have demonstrated that surgical trauma to the skin failed to promote experimental metastases in mice. Based on their studies, they have proposed that wounding creates a micro environment suitable for cell attachment, but the cells must be equipped to interact with the injured tissue. The frequency of tumour implantation at trauma sites is reduced significantly by pre- exposing the cells to fibrinogen, but not when they are pre-exposed to fibrinogen depleted plasma, serum or albumin. This indicates that fibrinogen, fibrin or related proteins, may be involved in facilitating tumour cell attachment to wounds (Murthy *et al,* 1991). The initial clot, formed in the wound, provides a structure to which tumour cells may attach and proliferate. This structure, a complex macromolecular matrix, contains multiple adhesion molecules to which tumour cells with appropriate receptors may bind. After the cells attach to the extracellular protein, other factors such as fibrin, protect the tumour cells from host defence system and also provide an initial source of nutrition (Murthy *et al,* 1989). It has also been shown that release of growth factor stimulated tumour growth after surgery. The same reparative process involved in wound healing apparently contributes to tumour attachment and growth (Jones & Rous, 1914; Lennard *et al,* 1985; Little *et al,* 1993).

The effect of incision size on tumour growth has not been evaluated. There is circumstantial evidence from the reported cases of wound recurrence so far following laparoscopic surgery, that these small wounds made during laparoscopy may differ in their ability to support tumour growth when compared to large wounds:

(a) When wound recurrence occurred in a patient whose operation was converted to an open laparotomy from a laparoscopic procedure, only the laparoscopic wound was involved although the larger laparotomy wound was made during the same operation (Jacobi *et al,* 1995).

(b) When wound recurrence occurred after laparotomy for colon cancer in a patient who recently had undergone a laparoscopic operation for benign disease, it was at the trocar site used for the previous laparoscopy and not the laparotomy incision (Ugarte, 1995).

(c) Ovarian cancer which seldom ever re-occurs in a laparotomy incision, can develop rapidly in a paracentesis needle tract (Kruitwagen *et al,* 1996).

The vulnerability of the laparoscopic wound to tumour recurrence may be due to factors in the laparoscopic environment, or inherent in the wounds due to alterations in the immune response or healing pattern of these small wounds.

The laparoscopic wound may present a localised area of trauma and ischaemia. Tissue ischaemia is known to release vasoactive substances from the affected tissue and reduce local defence mechanisms. An impairment of abdominal wall tissue perfusion during CO_2 pneumoperitoneum has been demonstrated to occur. Blood flow to the abdominal wall is affected by CO_2 insufflation due to the longitudinal stretch, which causes a narrowing of the diameter of the blood vessels supplying the abdominal wall (Schilling *et al,* 1997). Any of these factors, or a combination of these factors, may influence the development of implantation metastases at the port site wounds.

The successful implantation and growth of tumour cells at trauma sites depends on the complex interplay of: chemotactic attraction of tumour cells; adhesion molecules in the wounds; appropriate receptors for the adhesion molecules and tumour cell growth factors (Murthy *et al,* 1991).

Macrophages appear at the site of injury within 48-72 hours after wounding (Barbul, 1992). Tissue macrophages have been shown to play a critical role in the wound healing process (Redmond *et al,* 1992). Macrophages are essential for normal wound repair and many of their effects on healing are likely to be mediated by the secretion of cytokines (Murphy *et al,* 1988). These cytokines also participate in the early inflammatory response to wound healing. Through the generation of bioactive substances, macrophages orchestrate the complex process of cellular proliferation and functional tissue generation within wounds. The presence of macrophages is essential for the initiation and maintenance of wound fibroblast activity (Redmond *et al,* 1992).

Fixed tissue macrophages play a fundamental role in cellular and humoral immune responses (Johnston, 1988). Macrophages are located at strategic anatomic locations throughout the body and are critical in the elimination of pathogenic organisms and malignant cells. These cells are phagocytosed in opsonised and non-opsonised forms. Macrophages have the potential to carry out phagocytosis, antigen presentation, cell lysis and cytokine production. Macrophages maintain the cell-mediated immunity function by cytokine expression (IL-6 and TNF-α). Cell migration, cell killing, enzyme activity and phagocytosis, are all mechanisms which are called into play in combating pathogens and tumour cells (Redmond *et al,* 1992).

In this study, both the wounds in the animals that underwent laparoscopy, one which was used for the laparoscopic cannula and the other used for the venting cannula, exhibited the same attenuated macrophage response. In the earlier study described in this thesis using the solid tumour model (Chapter 3), it was noticed that the pattern of wound metastases did not have a predilection for any particular wound, when the

laparoscopy and the venting wound were compared. The nature of the attenuated immune response seen in both the wounds in this study, indicates that this may be a contributory factor for the observation seen in that study.

The difference in cellular activity of the wound could change over the period of the whole healing process, so further studies are necessary to characterise the changes that occur during different time periods of the healing process. However, the migration of macropahages to the wound are maximal by 48 hours. Wounds also produce growth factors which may have an influence on the healing process, as well as facilitating implantation of tumour cells. Factors such as cell adhesion, angiogenic factors and cytokine expression may play a role in the tissue attachment, migration, invasion and metastasis of malignant cells (Fujita *et al,* 1992). These factors should be studied further.

In summary, this preliminary study raises the possibility that the small size of the laparoscopic wound with its attenuated macrophage response may be a factor facilitating implantation of free tumour cells.

CHAPTER 13 METABOLIC EFFECTS

13.1. Metabolic Effects of CO_2 Insufflation on Surface pH in the Peritoneum of the Rat Model.

Introduction

The physiologic and metabolic effects of CO_2 pneumoperitoneum have been studied in humans and experimental models (Safran & Orlando, 1994; Ortega *et al,* 1995; Wolf *et al,* 1995). There is hypercarbia and an increase in arterial pCO_2 levels during CO_2 insufflation (Schob *et al,* 1996). However, the changes in the peritoneal cavity and on peritoneal surface is less clear. It has been postulated that CO_2 in the peritoneal cavity under high pressure can lead to increased absorption of CO_2 and also cause a local effect, by changing the pH of the peritoneum (Volz *et al,* 1996). This change in pH may alter the immunological and cellular integrity of the peritoneum. The magnitude of change in the pH on the peritoneal surface has not been clearly elucidated.

Aim of the study

The aim of this study was to measure the pH profile of the peritoneum in response to laparotomy, laparoscopy with CO_2 and gasless laparoscopy in the DA rat.

Material and Methods

pH Measurements

A paediatric monocrystant antimony probe with a single sensor was used (Monocrystant Model G 91-9011, Synectics Medical, Sweden). The probe with a diameter of 1.5 mm and was narrow enough to be passed through the channel of the micro laparoscopic port. The probe had a response time of less than 30 seconds. This was used in conjunction with an external Ag/ AgCl, ECG type reference electrode. The

reference electrode with electrode gel (Hellige, Germany) was placed in contact with a shaved area on the dorsal skin surface of the rat. The probes were connected to a portable digital recorder, which gave a constant visual display of the pH recorded by the probe and there was the facility for continuous recording and storage of data (Digitrapper - Model Mk II Gold- Synectics Medical, Sweden). The viability of the pH probe was checked with bench testing in buffers pH 7.01 and pH 1.07 (Synectics Medical Inc, Texas). The pH reading displayed on the digitrapper was noted before and after each operative procedure.

Three groups of rats underwent either laparotomy or laparoscopy with CO_2 pneumoperitoneum or the gasless laparoscopic technique. All the studies were performed under general anaesthesia administered using a tight fitting mask, through which a combination of halothane and oxygen, supplemented with nitrous oxide was used. The animals were monitored for any respiratory distress.

Laparotomy

Six rats underwent a conventional laparotomy through a 3 cm long midline abdominal incision. The surface pH of peritoneum was measured. The abdominal cavity was kept open for 30 minutes and at the end of that period, the peritoneal surface pH was measured again.

Laparoscopy using carbon dioxide pneumoperitoneum

Six rats underwent laparoscopy facilitated by conventional CO_2 pneumoperitoneum. Pneumoperitoneum was initially established using a Veress needle sited through a small stab incision to the right of the umbilicus. A disposable mini-laparoscopy cannula (Imagyn Medical, Laguna Niguel, California, USA) was then introduced through the needle to provide access for a 2 mm diameter mini-laparoscope (Imagyn Medical) attached to a conventional laparoscopic camera. CO_2 gas was insufflated at a flow rate of 0.2 L/ min and at a maximum pressure of 4 mmHg.

A 20 gauge intravenous cannula was next introduced through the left upper quadrant abdominal wall. An 18 gauge intravenous cannula was introduced into the abdominal cavity through the left lower quadrant abdominal wall and left 'open' to air. This acted as a venting cannula, ensuring a constant circulation of carbon dioxide through the peritoneal cavity. Pneumoperitoneum was maintained for 30 minutes. The pH of the peritoneal surface was measured at 1 minute after the establishment of the pneumoperitoneum and again 30 minutes later, before the insufflation of CO_2 was stopped. This was achieved by passing the antimony pH probe through the mini-cannula and angulating the mini-cannula to face the lateral peritoneal wall, to touch the peritoneal surface.

Laparoscopy using a gasless technique

In this group, 6 rats underwent laparoscopic surgery using an identical protocol to the conventional laparoscopy group, except CO_2 insufflation was omitted and a gasless technique was used. A Veress needle with a very brief period of CO_2 insufflation, was used for the introduction of the first trocar. This was to ensure the intraperitoneal positioning of the trocars and ports. A working space in the abdominal cavity was maintained by suspending the anterior abdominal wall from a wire frame, using multiple 3-0 silk sutures passed through the abdominal wall skin and anchored to the frame. A 20 gauge and an 18 gauge intravenous cannula were introduced separately into the left upper quadrant. This cannula was then closed to air while the left lower quadrant port and the laparoscopic port were left open to atmospheric air. After the ports were left open to the atmosphere for one minute, the pH probe was introduced to take the initial reading. All ports were left in place for 30 minutes, while the abdominal wall remained suspended. The pH of the peritoneal surface was measured at 1 minute after releasing the CO_2 and after 30 minutes at the end of the procedure, the antimony pH probe was passed through the mini-cannula using the same method as in the laparoscopy group.

Statistics

The data was analysed using the analysis of variance (ANOVA) test and significant differences between groups was assessed by the Tukey-Kramer multiple comparison test.

Results

The initial reading from both the laparoscopic groups suggests that even a short period of CO_2 insufflation can cause acidosis on the peritoneal surface, because in both of these groups there was only a short duration of pneumoperitoneum before the pH readings were taken. Thereafter the pneumoperitoneum was continued in the CO_2 group, while the gas insufflation was discontinued in the gasless group.

The continuing insufflation of CO_2 in the laparoscopy group produced a progressive acidosis of the peritoneal surface, as evidenced by the falling pH recorded by the pH probe. The initial acidosis in the gasless group recovered to the same level as the pre operative level in the laparotomy group.

TABLE 13.1

PERITONEAL SURFACE pH MEASUREMENTS

Start of Surgery (T = 1 minute)	Median pH	Confidence Interval
Laparoscopy with CO_2	6.4	6.1, 6.8
Gasless Laparoscopy	6.5	6.2, 6.8
Laparotomy	7.1	6.6, 7.4

End of Surgery (T = 30 minutes)		
Laparoscopy with CO_2	6.0	5.6, 6.4
Gasless Laparoscopy	6.8	6.4, 7.1
Laparotomy	7.2	6.9, 7.4

pH at 1 min and 30 min after start of each procedure

Statistical test	P value
One way analysis of Variance (ANOVA)	$P < 0.0001$
Tukey -Kramer Multiple comparison test :	
After Laparoscopy + Gas *vs* After Gasless Laparoscopy	$P < 0.001$
After Laparoscopy + Gas *vs* After Laparotomy	$P < 0.001$
After Gasless Laparoscopy *vs* After Laparotomy	$P > 0.05$ i.e. N S

TABLE 13.2

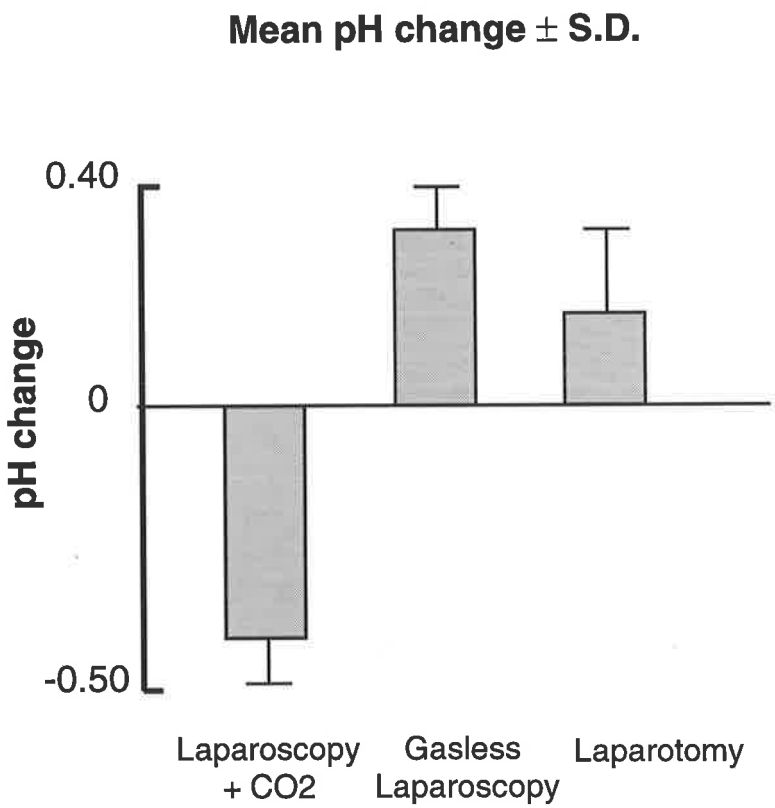
NET pH CHANGE
(median values)

Surgery Interval (T30 - T1 minute)	Median pH	Confidence Interval
Laparoscopy with CO_2	- 0.4	- 0.46, - 0.3
Gasless Laparoscopy	0.3	0.2, 0.3
Laparotomy	0.1	0.05, 0.2

pH difference for each procedure

Statistical test	P value
One way analysis of Variance (ANOVA)	$P < 0.0001$
Tukey -Kramer Multiple comparison test :	
After Laparoscopy + Gas *vs* After Gasless Laparoscopy	$P < 0.001$
After Laparoscopy + Gas *vs* After Laparotomy	$P < 0.001$
After Gasless Laparoscopy *vs* After Laparotomy	$P > 0.05$ i.e. N S

FIGURE 13.1



When the net change in pH between the time periods of 1 minute and 30 minutes was calculated, there was a significant sustained acidosis in the laparoscopy with CO_2 group. In both the gasless laparoscopy and the laparotomy group, there was an increase in pH.

Discussion

The results of the present study show that there is a significant reduction in the surface pH of the peritoneum during insufflation with CO_2. There was a lower pH in the gas and gasless group when compared to the open group to start with and this can be explained by the fact that CO_2 was insufflated in the beginning to ensure that the ports were intraperitoneal and that there was room for the pH probe to pass. There was a one minute interval before the pH reading was taken in the gas and gasless group, but this interval, may not have been long enough for the pH in the gasless group to return to the baseline. However, the net change in pH in this study shows a significant acidosis. In the laparoscopy with CO_2, there was a continuing acidosis following the maintenance of the CO_2 pneumoperitoneum. While in the gasless group, the acidosis following the initial instillation of CO_2, recovered to almost the same level as the peritoneal pH at the start of the operation in the open group. This is also indicative that physiological compensatory mechanisms are involved to neutralise this pH change due to the presence of CO_2.

The peritoneum consists of a monolayer of flat mesothelial cells loosely linked by light junctions and desmosomes. The basement membrane and dense connective tissue are adjacent. The surface consists of multiple microvilli and is covered by glycoprotein and glycosaminoglycanes. These structures produce a negative charge at the surface, preventing attachment and adhesion of intra-abdominal organs (Tsai *et al,* 1995). Implantation of disseminated tumour cells might be closely related to some local adsorptive factor in the normal and injured peritoneum (Miyazaki, 1986; Yonemura *et al,* 1996). When a lesion is present, there is an interaction between the surface and the extracellular matrix, favouring tumour cell adhesion. The exposure of basement membrane, induced by a mechanical lesion or stretching of the mesothelium, allows attachment of malignant cells to extracellular matrix proteins such as lamina, fibronectin and collagen. This produces changes in the intercellular integrity, enabling attachment, proliferation and finally infiltration of tumour cells and is a potential mechanism for tumour cell adhesion at the mesothelial surface (Volz *et al,* 1996).

Volz *et al* conducted a detailed study of the effect of CO_2 pneumoperitoneum in a pig model. They detected that the pneumoperitoneum caused a significant acidosis in the peritoneal drainage area. CO_2 pneumoperitoneum produces severe acidosis, not only on the peritoneal surface, but also in the underlying connective tissue. Severe acidosis in connection with an elevated intra abdominal pressure, may induce the changes in the different cell layers of the abdominal wall (Volz *et al,* 1996). A disturbance in the electrical surface charge has also been postulated to occur, because of CO_2 induced acidosis (Volz *et al,* 1996).

Many substances and stimuli are thought to affect the structural and immune functions of the peritoneum (Pancorbo *et al,* 1994; Tsai *et al,* 1995). Repeated exposure of cultured peritoneal mesothelial cells to lactate has induced cell injury and death (Shostak *et al,* 1996). A high lactate concentration in combination with a low pH has been demonstrated to result in a suppression of all peritoneal macrophage function. Acidic peritoneal dialysis solution suppresses all measured peritoneal macrophage function. This also causes decreased cell growth and a reduced rate of regeneration of cultured mesothelial cells *in vitro* (Shostak *et al,* 1996). Volz *et al* in their study, took smears from the peritoneal cavity at the start and finish of the insufflation and demonstrated that although the first smear at the start of the procedure showed regular mesothelial cells, the post operative smears showed a high infiltration of granulocytes into the peritoneal surface and fibrin degradation products (Volz *et al,* 1996). Necrosis and exfoliation of the mesothelial layer of the peritoneum is known to develop in the acute inflammatory stage of peritonitis (Tsai *et al,* 1995). An increase of peritoneal pH stopped this septic process in the peritoneum and improved survival in experimental model (Imhof *et al,* 1987). Mesothelial cells of the peritoneal cavity become round and separated resulting in the exposure of the underlying connective tissue, metastatic tumour cells are found to adhere to the naked areas of the sub mesothelial connective tissue (Yonemura *et al,* 1996).

Carbon dioxide is known to cause acidosis of the peritoneal cavity and the resultant acidity can act as a strong irritant to the peritoneal surface (Volz *et al,* 1996). This irritant effect may produce changes which may contribute to the facilitation of peritoneal spread and wound recurrence of tumour (Volz *et al,* 1996). In a study conducted by Iwanaka *et al* in mice, the changes in macrophage TNF-α production following CO_2 insufflation was significantly less than in the open group (Iwanaka *et al,* 1997).

The metabolic environment associated with low pH leading to increased lactate production, has been suggested to lead to activated macrophages producing angiogenic factors which enhance tumour growth. Lactic acid itself can act as a metabolic signal to activate macrophages to release angiogenic factors. An anaerobic acidic environment may itself be the signal responsible for initiating a cascade of macrophage infiltration and high TNF-α production (Stovroff *et al,* 1989).

One of the postulated metastatic routes of the peritoneum is by the adhesion to the naked connective tissue, exposed after the shrinkage of the mesothelial cells. Implantation of disseminated tumour cells might be closely related to some adsorptive factor in the injured peritoneum (Miyazaki, 1986; Fujita *et al,* 1992; Yonemura *et al,* 1996).

Volz *et al* (1996) in their study of the physiological alterations caused by CO_2, documented an increase in the release of catecholamines during induction and desufflation of the pneumoperitoneum. This may be primarily due to irritation and toxic damage to tissues, but could also be attributable to the stretch caused by the pneumoperitoneum or the acidosis, which exists during this manipulation. Acidosis being a strong irritant to the peritoneal surface, lead Volz *et al* to postulate that the catecholamine release was in response to this acidosis in the peritoneum and adjacent tissue layers (Volz *et al,* 1996).

There is no clear understanding of the effect of CO_2 directly on the growth and metabolism of cells. CO_2 may acidify the milieu of cancer cells and this may affect the tumour growth. Nduka *et al*, 1997, investigating the effect of CO_2 pneumoperitoneum on *in vivo* tumour growth, found that CO_2 exposure resulted in significant acidification of the cancer cell micro environment, with significantly enhanced tumour growth both intra peritoneally and at the wound.

In a study by Jacobi of *in vitro* tumour growth, they found that *in vitro* tumour growth was significantly increased after incubation with CO_2 and air. They also found the growth of tumour in subcutaneous tissue was higher with CO_2 insufflation and attributed this to the higher levels of CO_2 in the subcutaneous tissue (Jacobi *et al,* 1997a).

An important prerequisite of tumour cell seeding is impaired host defence mechanisms (Lundy *et al,* 1979). It is known that the effective function of macrophages, the major cell for host defence against intraperitoneal tumour cells, is reduced by surgery (Redmond *et al,* 1992). Whether a CO_2 pneumoperitoneum affects macrophage function is not known. Previous experimental work indicates that exposure to endotoxin (Watson *et al,* 1995b) and acidosis may alter or impair peritoneal cellular response. The results of this study support the concern that as a consequence of the metabolic effects produced by CO_2, intraperitoneal metastases are promoted by a CO_2 pneumoperitoneum. The extent to which these changes contribute to the development of peritoneal implantation and port site tumour recurrence associated with laparoscopic surgery, needs to be evaluated by further studies.

CHAPTER 14

14.1. Discussion and Conclusions

The impact of Laparoscopic surgery

The advantage of a reduced postoperative recovery period after laparoscopic surgery holds a great attraction to patients, surgeons and health care administrators. The momentum, propelling the fast moving pace of the application of laparoscopic surgery, is partly due to increased patient demand and economic forces.

The application of the laparoscopic technique for the treatment of benign diseases has expanded to include the treatment of malignant disease, in the abdominal and thoracic cavities (Shimi *et al,* 1992; Trus & Hunter, 1997). There are reports of a growing number of laparoscopic procedures being performed for intra-abdominal malignancies (Thibault & Poulin, 1995; Schirmer, 1996), including malignancies of the thoracic cavity (Boutin & Rey, 1993; Landreneau *et al,* 1993). In particular, laparoscopic surgery for colonic carcinoma is gaining popularity in many centres around the world (Fingerhut, 1995; Ota, 1995).

Laparoscopic examination of the abdominal and thoracic cavities for the diagnosis and staging of malignancy is a very attractive alternative to an open procedure, because it reduces the morbidity and avoids an unwanted laparotomy, especially in patients with an inoperable malignancy. However, the accelerated application of laparoscopic surgery for the treatment of malignant diseases has had a setback, with increasing reports of port site dissemination of tumour (Cava *et al,* 1990; Barsoum & Windsor, 1992; Aractingi *et al,* 1993; Berends *et al,* 1994; Dixit *et al,* 1997).

Port site metastases - the current scenario.

The first report of port site metastases in the literature occurred in a patient with an ovarian malignancy, who underwent laparoscopy and subsequently developed tumour at the port site wounds (Döbrönte *et al,* 1978). Since there have been further such reports (Martinez *et al,* 1995; Johnstone *et al,* 1996). There are also reports of port site recurrence following laparoscopic surgery for carcinoma of: the colon (Akle, 1996), stomach (Cava *et al,* 1990), gallbladder (Sailer *et al,* 1995), ovary (Gleeson *et al,* 1993), pancreas (Siriwardena & Samarji, 1993), liver (Keate & Shaffer, 1992) and urinary bladder (Andersen & Steven, 1995). This phenomenon has also been observed after thoracoscopic resections for carcinoma of the lung (Buhr *et al,* 1995). An increasing number of implantation metastases to chest access wounds are being reported in association with the increased popularity of the video assisted thoracoscopic procedure (Buhr *et al,* 1995). Downey *et al,* 1996, reported a series of 21 cases, where dissemination developed following video assisted thoracoscopic surgery. This has occurred following the handling of tumours, of both primary and secondary in origin, in the lung. The metastases were of many tissue types, although adenocarcinoma was the predominant variety.

Numerous cases, amounting to almost one hundred, have been reported since the first report of port site metastases (Döbrönte *et al,* 1978; Nduka *et al,* 1994; Martinez *et al,* 1995; Wexner & Cohen, 1995; Johnstone *et al,* 1996; Kruitwagen *et al,* 1996). The magnitude of this problem is still not clear. The number of cases of laparoscopic surgery performed and the number which have developed port site recurrence or are likely to, is unknown. It is likely to become a problem of focus in the future with the ever increasing number of laparoscopic procedures being performed for malignant disease. In a retrospective analysis of patients' port site dissemination following laparoscopy for ovarian malignancy, the incidence was estimated to be 16% by Kriutwagen *et al,* 1996. Wexner *et al,* 1995, in a review of this problem following laparoscopic colectomy, reported that the incidence may be above 4%. In a report by Prasad *et al,* 1994, the incidence of port site metastases following laparoscopic assisted

colectomy was estimated to be 4%. The increasing number of reports of tumour recurrence in access wounds for laparoscopic surgery, has prompted many surgeons to pause and question the safety and applicability of laparoscopic techniques to the treatment of malignant disease (Kmiot & Wexner, 1995; Downey *et al,* 1996; Johnstone *et al,* 1996; Volz *et al,* 1997).

Clinical features of port site recurrence and its influence on morbidity

The impact of port site metastases on survival is not clear. There is currently disagreement between surgeons about the significance of metastases arising in trocar wounds following laparoscopic surgery for malignant disease. A body of both clinical and laboratory evidence now exists, which supports a position of caution with respect to the application of laparoscopy to malignancy (Nduka *et al,* 1994; Wexner & Cohen, 1995). It has been suggested that wound metastasis occurs following the direct contamination of laparoscopic instruments with tumour cells during the laparoscopic manipulation of malignancies, with resultant spread to abdominal wall wounds occurring by direct transfer when trocars and instruments are withdrawn from the peritoneal cavity (Hewett *et al,* 1996). If this is the case, then wound metastases may be a localised problem and barrier strategies which protect the wounds during laparoscopic surgery may be sufficient to overcome this particular problem. It is also possible that cells are moved around by the insufflation gas used for laparoscopy and transferred to wounds, without any direct physical contact occurring with contaminated instruments (Jones *et al,* 1995). If this is occurring, then the phenomenon of port site metastasis may be a sign indicating generalised tumour spread due to possible adverse effects of the laparoscopic environment and may have a grave prognosis.

Information from the cases reported in the literature so far reveal that port site metastases may develop as early as one week following surgery and occur as late as 26 months (Johnstone *et al,* 1996). In a review of all the reported cases by Martinez *et al,* they report a period of 9 weeks in gynaecological malignancies and 8 months in digestive tumours, as the mean interval for development of port site metastases

(Martinez *et al,* 1995). These lesions present as hard, painful nodules at the previous placement site of one or more of the laparoscopic cannula (Nduka *et al,* 1994). Although most corresponded to advanced disease, there were also cases of early disease (Prasad *et al,* 1994; Shepherd *et al,* 1994). The development of this complication is bound to adversely affect the survival and quality of life of these patients, especially those who had early disease.

Laparoscopic surgery for malignancy, although very attractive in terms of significantly reduced wound related morbidity and an early recovery, will be a cause of increased suffering if it enhances the possibility of increased tumour spread. This could result in decreased survival and quality of life for the patient. It will be unacceptable if laparoscopy hastens the disease progression in patients with early disease, which may have been curable by conventional surgery (Cotlar *et al,* 1996).

Port site recurrences are a difficult management problem. They may require extensive chest or abdominal wall resections and often complex repairs. These interventions add to the morbidity associated with the care of the primary disease. The report of Frey *et al,* 1995, demonstrates that the development of port site recurrences are generally a poor prognostic indicator. No matter what benefits to the patient may exist in terms of reduced post-operative pain or shortened hospital stay, any technique should be used with caution if the modality appears to cause an increased opportunity for cancer spread.

Possible aetiologic factors

A range of possible explanations for the occurrence of port site metastases have been proposed and it is likely that the aetiology is multifactorial, with many possible contributing factors leading to the development of this problem. The most feasible explanation is that the increased incidence of this phenomenon is due to direct contamination by the tumour cells on the port site wounds. This could be through contaminated instruments or cannulae (Keate & Shaffer, 1992), which are used for laparoscopic surgery. By nature of the technique involved in laparoscopic surgery, tactile sensory feedback is lost and this may result in the increased handling or disturbance of tissues. This could lead to increased spillage of tumour cells. There is also an increase in contact between the port wound and the instruments, because of the constant 'in and out' movement of the instruments during procedures. It is also possible that there may be direct implantation of tumour cells to the wound surface, when bulky specimens are removed through a very small incision (Alexander *et al,* 1993; Sandor *et al,* 1995). However, the above mechanisms of direct contamination or implantation by instruments or ports carrying tumour on their surface, do not explain the incidence of all the port site recurrences.

The local factors within the port site wounds (being an area of epithelial breach, injury and possible ischaemia) may facilitate the implantation and growth of tumour cells (Jones *et al,* 1995; Wu & Mustoe, 1995). Murthy *et al,* 1989, have demonstrated that malignant cells implant preferentially in areas of recent injury and high cell proliferation due to healing.

The difference between a laparoscopic procedure and laparotomy are: the smaller size of the access wounds and the creation of a pneumoperitoneum by the introduction of gas under pressure into the peritoneal cavity. This creates the space needed by the surgeon, for adequate visualisation and for movement of instruments. Various factors are involved in the creation and maintenance of the laparoscopic environment which may play a role in the development of port site metastases. These

include: mechanical effects of gas under pressure (Bouvy *et al,* 1996b; Hubens *et al,* 1996), metabolic and immunological effects, due to the insufflating gas (Evrard *et al,* 1996), alterations in peritoneal micro circulation (Schilling *et al,* 1997), stretching of the abdominal wall and peritoneal lining (Volz *et al,* 1996) and release of catecholamine and vasoactive substances in response to peritoneal irritation or stretch (Volz *et al,* 1996).

Unexplained features of port site metastases

When carefully reviewing the cases reported in the literature so far, this phenomenon is intriguing for several reasons:

1. All the recurrences have not occurred at port sites through which the specimens were retrieved. Port site tumours have been reported at sites where direct contamination is unlikely and in cases in which the primary tumour was not manipulated. Watson reports the development of multiple abdominal wall metastases from a pancreatic carcinoma, in a patient following a laparoscopic gastroenterostomy and the primary tumour was not handled or manipulated (Watson, 1995).

2. The actual number of such recurrences is unknown. The reports from retrospective analyses of this problem available so far, have quoted an incidence ranging from 6.3 % - 21 %. If this is the true incidence, then it is higher than the reported incidence of wound metastases which is 0.68 % - 1 % following open surgery. The numbers of port site recurrence reported so far, may be just the "tip of the iceberg" (Berends *et al,* 1994; Wexner & Cohen, 1995).

3. It has been argued that the development of port site metastases is a reflection of the advanced stage or aggressiveness of the neoplasm. This factor, however, does not explain the development of port site recurrence in borderline ovarian tumours, which are known to have a low malignant potential (Shepherd *et al,* 1995) or in early carcinoma, such as Dukes A colonic carcinoma (Prasad *et al,* 1994).

4. Port site recurrence does not seem to be unique to any particular type of malignancy, although these reports were mainly associated with laparoscopic surgery for carcinoma of the colon (Akle, 1996), gallbladder (Sailer *et al,* 1995) and ovary (Gleeson *et al,* 1993). Recently, there have been reports of port site recurrence in gastric (Cava *et al,* 1990), pancreatic (Siriwardena & Samarji, 1993), oesophageal (Dixit *et al,* 1997), lung (Fry *et al,* 1995), urinary bladder (Andersen & Steven, 1995), prostate (Bangma *et al,* 1995) and lymphomas (Aractingi *et al,* 1993) following laparoscopy.

Strategies to investigate the possible mechanisms involved in port site metastases.

The gold standard to evaluate the efficacy and outcome of any therapeutic intervention is a randomised, controlled trial. A randomised trial to compare the outcome of laparoscopic surgery with open surgery, in a matched population of patients with malignant disease, will help to resolve this dilemma. However, such studies would be difficult given the generally low incidence of the phenomenon and hence a large number of patients would be required to enable statistical analysis. The findings will take even longer to be completed and reported in surgical literature. An alternative method to evaluate this problem is to use an animal model to reproduce conditions that exist in the human situation, in respect of the behaviour of the tumour and the similarity of the intervention technique (Fisher, 1983). Animal models have been used for a considerable period of time to understand behaviour of disease processes and physiological reactions to therapeutic interventions. These models have also been used for the study of malignant tumour behaviour and the response to therapeutic interventions. Some of these models have been used extensively to understand the metabolic response, development of metastases and the tumour behaviour after manipulation (Fisher, 1983).

Experimental Models to investigate the aetiology of port site metastases

Since the work on the model described in this thesis was started, other experimental models have been developed and used to understand the mechanism/s

which lead to port site metastases. Each of the models have their advantages and disadvantages.

Large animal models

Many investigators have used large animals, such as the pig (Allardyce *et al,* 1996; Hewett *et al,* 1996; Texler *et al,* 1997). The advantage of this model has been that the peritoneal cavity is similar in volume to the human and hence the volume and the flow patterns in the pig's abdomen may be similar. This factor may be useful to study intra-operative cell movement and migration. The pig has also been used to investigate the physiological changes during and after laparoscopy. However, studies performed using large animal models, such as the pig, suffer from several short comings.

Few cultured tumour cell lines have been developed for large animals and thus it is difficult to study *in vivo* tumour behaviour. Usually human cell lines are used and so only the short term effects of tumour spread can be studied. The natural history of implantation and growth cannot be extrapolated to the human situation with these studies, because these tumours do not normally originate or grow in the large animals.

Physiologic, immunologic and oncologic assays often require species specific reagents and unfortunately there are limited assays and reagents available for large animal models (Allendorf *et al,* 1997).

Small animal models

A few investigators have used hamsters, here too the disadvantage is that they have not used any native tumour cell line (Jones *et al,* 1995).

There has been a number of investigators who have used rats or mice to study the effect of tumour spread (Allendorf *et al,* 1995a; Bouvy *et al,* 1996b; Volz *et al,* 1996; Jacobi *et al,* 1997a). In some studies, strains with suppressed immunity and

human cell lines were used. Other studies used immunocompetent mice or rats, with carcinogen induced tumour cell lines. Since beginning this study, more small animal model work has emerged. The data presented in this thesis is the only work to date using a naturally occurring tumour and cell line native to an immunocompetent rat, which has been used to investigate this phenomenon. The advantages of these small animal models are:

- there are a variety of tumour cell lines which can be used in small animal models and so studies on natural history and survival can be conducted;
- the biochemical and immunological responses of the small animal models have been well characterised;
- species specific reagents for physiologic and immuniologic assays are readily available.

The main drawback of the small animal model is that the capacity of the abdomen is small and a smaller volume in these animals may not be readily comparable to the larger volume in the humans. The small animal needs a modified laparoscope and instruments for laparoscopic manipulation. Thus, it is technically more challenging to perform procedures efficiently and safely.

Whilst all that occurs in the rat cannot be extrapolated to the human situation, it provides a useful model to gain insight into the pathophysiology and results of manipulation of the laparoscopic environment.

Bench top models

Some investigators have used *in vitro* models using chambers or containers to simulate the peritoneal cavity (Thomas *et al,* 1996; Whelan *et al,* 1996). These are artificial environments when compared to the animal and even more so when compared to the human peritoneal cavity. These models cannot reproduce the natural melieu of

the living peritoneal cavity with possibly complex responses to the laparoscopic environment and manipulation. These man-made "peritoneums" have been used to study cell movement and distribution in response to gas insufflation. Apart from understanding the pure physical aspect of cellular or particulate movement with gas, very little else can be extrapolated to the human situation from these models.

Findings from studies presented in this work

Work presented in Chapter 2 of this thesis demonstrates the establishment of the Dark Agouti rat model of implantable abdominal wall tumour, which is consistent in its metastatic behaviour and growth. This model is amenable to laparoscopic and open surgery, thus eminently suitable for studying the effect of laparoscopy and manipulation of the laparoscopic environment.

The work described in Chapter 3 documents a very high rate of wound metastases in rats undergoing laparoscopic tumour laceration, compared to rats undergoing an identical procedure at open surgery. There was a five-fold increase in the presence of tumour in wounds of animals undergoing laparoscopy. Tumour was present in port wounds used for passage of the laparoscope, as well as that used for tumour laceration. This pattern of distribution of tumour cells suggests there is some factor inherent in the laparoscopic procedure or environment, rather than direct implantation of tumour from a tumour laden instrument or specimen extraction. This study also demonstrated a larger increase in the size of the primary tumour following laparotomy, when compared to laparoscopy. The differential growth of primary tumours in this study supports the observation of others (Allendorf *et al,* 1995a; Allendorf *et al,* 1995b), who have postulated this to be due to the beneficial effect of laparoscopic surgery when compared to laparotomy. Evidence from this study suggests that this benefit may be nullified by increased spread of tumour to wounds.

The results of a study by Jones *et al* (1995) using washed human colon cancer cells, injected as a cell suspension into the peritoneal cavity of hamsters (rather than an implanted tumour), also revealed a greater incidence of metastasis to abdominal wall wounds following laparoscopy. Although in the present study none of the animals in whom the tumour capsule was breached developed metastases, this finding may not hold true in clinical situations, because free cells may be present in the peritoneal cavity of patients with malignant disease even before tumour is breached or manipulated. The

results of the current study provides evidence that wound metastases are increased following laparoscopic manipulation of malignant tumours. This has serious clinical implications which must be addressed urgently.

In the next study, described in Chapter 4, using the solid tumour model, it was demonstrated that the presence of CO_2 pneumoperitoneum played an important role in the development of port site metastases. The elimination of CO_2 insufflation by performing gasless laparoscopy reduced the incidence of port site tumour recurrence to the same level as that of open surgery. Growth of the 'primary' tumour following laparoscopy, with and without gas was similar. Others have demonstrated reduced primary tumour growth following laparoscopy in animal models and have postulated beneficial immunological effects of laparoscopic surgery, compared to open surgery (Allendorf *et al,* 1995a). Earlier work confirmed the observation that tumour growth is less following laparoscopic surgery, when compared to equivalent open techniques. This effect is probably related to wound size alone and not some other factor inherent in the laparoscopic environment. When CO_2 insufflation was eliminated, by using a gasless laparoscopic technique, the port site metastasis rate was significantly less, resembling the incidence of wound metastasis following laparotomy in this model. Although Bouvy *et al* (1996b) have argued that laparoscopy is beneficial and reduces the tumour growth, their study also concluded that CO_2 produced increased tumour implantation and growth, when compared to gasless laparoscopy.

Volz *et al,* 1996, using a nude mouse model, arrived at the same conclusions when they studied the pattern of spread of intraperitoneally injected tumour following laparoscopy with CO_2 and gasless laparoscopy. Findings from this study may have major clinical importance in the fact that gasless laparoscopy, by eliminating carbon dioxide insufflation, may reduce the incidence of wound metastases.

The next study described in Chapter 5 documents the findings on an alternative model. The free cell model was developed as an alternative to the solid tumour model.

The aim of this study was to investigate the potential effect of CO_2 insufflation on tumour implantation patterns, rather than investigating if there was increased likelihood of tumour implantation in individual animals. A large enough inoculum was used intraperitoneally to ensure tumour growth in all the animals. A standard protocol of tumour cell dose and site of intraperitoneal instillation was used in all the animals. Although the number of free cells introduced into the animals' peritoneal cavity was in excess of that used by other workers and what may be found in the clinical setting, this study provided useful information about the possible effects of CO_2 insufflation on free tumour cells, which are present or liberated into the peritoneal cavity. Tumour redistribution and spread to all sectors and all port sites occurred in the majority of animals in the CO_2 insufflation group. In contrast, tumour implantation in the other two groups (laparotomy and gasless laparoscopy) was predominantly near the site of introduction of the tumour suspension. A similar pattern was seen for the spread of tumour to the surgical access wounds. This suggests that the insufflation of CO_2 gas during laparoscopy, leads to more widespread tumour deposition and growth, compared to gasless laparoscopy and laparotomy. However, this study has not clarified whether this phenomenon is due solely to the physical effects of the insufflatant gas, or whether this phenomenon is due to possible CO_2 induced alterations in tumour metabolism, or an altered pH environment of the peritoneal cavity. Volz *et al*, 1996, using a similar method of intraperitoneal injection, noted that CO_2 cause an increased implantation rate and growth of tumour.

Bouvy *et al* (1996a) using a different protocol to investigate the immune response, also observed that the control group with CO_2 had more tumour growth, than the control animals which underwent only anaesthesia. The mechanism by which CO_2 influences this growth requires further investigation. Jones *et al* (1995) in their work have noted that the presence of CO_2 enhances the implantation and growth of tumour, although the mechanism is not clear. Jacobi *et al* (1997a) in their study have demonstrated that tumour cell growth was increased significantly following incubation with CO_2. The results from this study add weight to existing evidence, which suggests

that wound metastases and tumour spread is more likely to occur following laparoscopic procedures for malignancy when using CO_2 insufflation.

In the next study described in Chapter 6 using the same model, but using only the venting hole without the cannula, it was demonstrated that CO_2 escaping through an open wound, produced a significant increase in the implantation of tumour within the abdominal musculature. There was also a significant increase in the incidence of tumour found in the subcutaneous tissue of port sites. This gave support to the hypothesis that the circulating gas is able to transport cells within and beyond the peritoneal cavity. Bouvy *et al* (1996b), argued that the incidence of port site metastases is due to cells being transported to the port site by leaking gas at the port site. Hubens *et al* (1996), noticed that the implantation of tumours was in the subcutaneous plane along the port site in a rat model, while Allardyce *et al* (1996) noticed increased contamination along working ports. All these investigators have postulated the same mechanism: leaking gas around the ports, transporting cells to the port site. Jaccobi *et al* (1997a) in a recently published report, reported an increase in tumour growth in the subcutaneous tissue following CO_2 insufflation in a rat model, where tumour cells were injected intraperitoneally and subsequently underwent insufflation with CO_2. Wounds were created by the introduction of trocars. They also noticed that subcutaneous tumour growth was promoted after laparoscopy with CO_2. Thus there may be a combination effect of the physical transport of cells and a direct metabolic effect on the tumour, or surrounding tissue by CO_2. However, Hewett *et al* (1996) and Whelan *et al* (1996) have postulated that port site metastases occur by contamination of ports, either by direct contact with tumour or contaminated instruments.

In the third study using the principle of the free cell model, described in Chapter 7, a set-up of cross circulation between two animals' abdominal cavities was achieved by circulating the gas vented from one animal through a second animal. It was demonstrated that CO_2 gas is able to transport cells outside the peritoneal cavity. In these studies, the presence of wound metastases in the recipient rat occurred in most of

the rats undergoing carbon dioxide insufflation and none of the rats in the gasless laparoscopy group and provides additional evidence that insufflation is essential for the promotion of wound metastases. Care was taken during the studies to prevent siphoning of tumour cells from the donor rat to the recipient rat. Tumour nodules occurred at both the inflow and outflow port wounds in the recipient rats undergoing insufflation. A larger inoculum of tumour cells was introduced into the donor rats in these studies than that used in earlier studies, to maximise the likelihood of metastases developing. A longer latency period was used to maximise the likelihood of tumour growth being clinically evident in the recipient rats. This study demonstrates that with the liberation of a large number of tumour cells into the peritoneal cavity, combined with CO_2 insufflation and a significant gas leak, the necessary environment for clinically important port site metastases is created.

Studies by Whelan *et al*, 1996, however, have revealed conflicting results. Their studies used a quantity of tumour cells which was 1000 times smaller than that used in the current studies and insufflation was continued for only 10 minutes. Outcome differences may therefore reflect a dose and time dependent response relationship.

In a study on patients it was demonstrated that the smoke, which is drawn into the port site, contains clumps of viable cells (Taffinder & Champault, 1996). Knolmayer *et al* (1996) and Texler *et al* (1997) in their published abstracts of work with a pig model to study the movement of cells in the venting gas, have documented that aerosolisation of tumour occurs during CO_2 insufflation. The work reported in this thesis using a unique experimental set-up, is another experimental model that suggests laparoscopy with carbon dioxide insufflation transports tumour cells to laparoscopic access wounds and results in the growth of metastases. This effect appears to be prevented by the use of gasless laparoscopy. The precise mechanism for this phenomenon, however, is not clear.

Dynamic studies on cell movement and distribution are difficult to perform. Although aerosolisation or the transport of cells by the circulating gas, has been postulated by many, there is still considerable debate in the literature on the ability of gas to transport cells in a liquid medium or as an aerosol. It is often observed that during laparoscopic procedures, particulate matter and smoke circulate around the peritoneal cavity.

Work presented in Chapter 8 of this thesis reports a model for assessing cellular movement by tagging the cells with a radioisotope. This study used a suspension of tagged cells introduced into the peritoneal cavity and measured the activity in the venting outflow, after bubbling the venting gas through a test tube of phosphate buffered saline. This study was performed in two groups of rats, one group underwent laparoscopy with CO_2 and the other underwent laparoscopy without CO_2. This study demonstrated that there was a significant number of cells transported by the gas leaking out of the peritoneal cavity, through the venting cannula. The activity of the venting gas revealed that there was a significant quantity of cells in the gas vented from the peritoneal cavity of rats undergoing insufflation, versus either no cells or very few, when using gasless laparoscopy. Jones, Allardyce and Hubens (Jones *et al,* 1995; Allardyce *et al,* 1996; Hubens *et al,* 1996) who have investigated this problem, proposed the "chimney effect" to explain this phenomenon. The chimney effect is associated with the development of a venturi phenomenon, whereby the insufflation of CO_2 causes turbulence which displaces tumour cells. At the port sites, these cells are concentrated as a result of the leakage of CO_2 alongside trocar, leading to a high local gas flow at the trocar sites. This gas may contain an aerosol of viable tumour cells, which may be delivered to the port sites, resulting in tumour implantation, as reported by Bouvy *et al* (1996b).

The subsequent study detailed in Chapter 9, was an attempt to quantify the amount of cells dispersed by the circulating gas, by using a smaller quantity of suspension of free tumour cells tagged with radioactive isotope. In this study, the aim

was to enumerate and verify if there was any difference in the distribution of cells within the peritoneal cavity, following laparotomy and laparoscopy.

This study demonstrated that the pattern of cell distribution within the peritoneal cavity was different during laparoscopy when compared with laparotomy. However, there was no difference between the gas and gasless laparoscopic group. Allardyce *et al* (1996) used tagged cells in a pig model and found that there was no significant difference in cells deposited at the port sites, however cells were found at the ports which underwent maximum movement. This study did not provide information on the ultimate outcome of cell implantation and growth.

There is evidence from experimental animal and human studies, that changes in the peritoneal environment are necessary for successful implantation and growth of malignant cells. The studies described in Chapter 10, aimed to investigate the influence of alterations in the immune status and peritoneal environment on tumour spread through the peritoneal cavity. Two different scenarios were created by using tumour bearing rats or lipopolysaccharide injected rats. This study demonstrated that both the alteration in immunity and change in peritoneal environment adversely influenced the intraperitoneal tumour spread.

In Chapter 11, a study of the change in macrophage activity in response to CO_2 insufflation was conducted. Macrophage activity was estimated by measuring TNF-α production by macrophages. TNF-α production was measured in harvested macrophages from the peritoneal cavity. Macrophages were harvested after laparotomy and laparoscopy, with and without CO_2 insufflation. The findings from this study indicate that CO_2 insufflation causes a reduced macrophage TNF-α production when compared to laparotomy and gasless laparoscopy. The changes in the peritoneal macrophage activity, as an indicator of peritoneal immune function, have also been found by another investigator (Iwanaka *et al,* 1997).

Changes in peritoneum alone do not explain all the incidences of wound metastases, as tumour cell implantation seems to be facilitated by the presence of wounds. It has been suggested that active port sites are the most vulnerable, either because of more likelihood of gas leaking around them; increased trauma, or a difference in inflammatory and immune response. There is circumstantial evidence from case reports that small laparoscopic wounds may support tumour growth more than larger incisions used for open surgery.

The next study, in Chapter 12, aimed to assess the immune response in the laparoscopic wound, when compared the wounds in laparotomy. An immuno-histochemical study of the wounds was performed. An immune marker was used to identify the population of mature macrophages. This revealed that the population of macrophages in both the laparoscopic and venting wounds were significantly reduced, when compared to laparotomy incisions. As macrophages are an essential component of wound healing and the immune response process, this raises the possibility that there may be an immune suppression or reduced immune response in the laparoscopic wounds with CO_2 insufflation. This could be due to the fact that the incisions used in laparoscopy are small and hence elicit minimal immune and inflammatory response, and by virtue of this attenuated response, the tissue is more vulnerable for successful tumour cell implantation and growth.

In Chapter 13, the results from the study of peritoneal pH is described. This study was conducted to investigate if there were any metabolic changes produced by the CO_2 insufflation. Volz *et al* (1996) in their detailed study of the physiological response to CO_2 pneumoperitoneum in a porcine model, have recorded that CO_2 causes a significant acidosis of the peritoneal surface and adjacent tissue and that leads to a significant metabolic and immune response by the peritoneum.

It is known that an acidic medium is a potent irritant to the peritoneum. It has also been documented that an acidic solution in the peritoneal cavity can cause a significant depression of macrophage function (Shostak *et al,* 1996). There is preliminary evidence that the growth of tumour cells is enhanced in an acidic medium. The acidic environment and presence of lactate may be a possible factor in the development of port site metastases. The acidosis and role of CO_2 in tumour biology demands further study.

The results from the various studies presented here indicate that the development of port site metastases is multifactorial.

Current Evidence from Other Studies on Experimental Models

Bench top models

There has been considerable interest in the aetiology of port site metastases, resulting in an increase in the amount of data in the literature of experimental studies, most of which post date the work presented in this thesis. Evidence from experimental studies investigating the aetiologic factors for the development of wound recurrence have been controversial so far.

Thomas *et al* (1996) used a bench top model to investigate the pattern of cell distribution after CO_2 insufflation. This was a rigid cavity, into which a free cell suspension could be placed and gas vented through a port site, or an instrument introduced to agitate the free cell suspension. They concluded that the distribution of cells was from direct contamination through instruments, which had cells attached to them.

Whelan *et al* (1996) used a similar bench top model along with a balloon attached at one end of the chamber, to mimic the peritoneum in the human. They insufflated air through this arrangement, after placing a cell suspension at the bottom of the container. The venting gas was collected in culture media to investigate the presence of cell growth. There was no evidence of aerosolisation in this study, but with cells placed in the balloon, rapid desufflation of the balloon produced a transfer of cells from the balloon to the container.

Both of these studies do not take into account the complex interactions of peritoneal surface and circulating cells. These studies also do not allow for the nature of cell surface and peritoneal surface charge interactions. The metabolic and immune changes on the peritoneal surface could not have been duplicated and hence may not represent the situation in a living organism.

Studies on small animal models:

Effect of CO_2 laparoscopy on growth of primary tumour

Allendorf *et al* (1995b) conducted two studies to investigate the effect of laparotomy and laparoscopy on tumour growth. They conducted two experimental studies with two groups. In each study, one group of rats underwent laparotomy and the other group underwent laparoscopy. In the first experiment, an intradermal injection with a low inoculum of mouse mammary adenocarcinoma cells was performed and then the rats underwent either laparotomy or laparoscopy. In the second experiment, the rats were subjected to an intradermal injection of a higher inoculum of tumour cells and then underwent either laparotomy or laparoscopy. The number and size of the tumours in both groups were assessed by palpation and after excision, at the end of 14 and 30 days. This study revealed that at the end of day 14 and 30, there was a significant increase in the number and size of tumours in the laparotomy group when compared to the laparoscopy group (Allendorf *et al,* 1995b). The tumours were more easily established after laparotomy, than after laparoscopy. Allendorf *et al* attributed this to the relative immunosuppression after laparotomy. There is evidence that the degree of physiological response and immune suppression depends on the magnitude of the surgical procedure.

In a subsequent study, Allendorf *et al* (1995a) used a larger number of animals with a modified protocol. In this study they conducted three experiments with animals randomised to three groups in each experiment i.e. a control group with only anaesthesia, a laparotomy group and an insufflation group. All the rats underwent intradermal injection of tumour cells before one of the procedures. In the first group, the growth was observed at the end of 3, 6, 10 and 12 days; in the second experiment, the tumours were excised and measured; in the third experiment the tumour growth was observed for 30 days. This study revealed that there was a significant increase in the number and size of tumours in the laparotomy group, when compared to control and laparoscopy group.

The findings from this study indicate that this difference may be due to the reduced stress in animals undergoing laparoscopic surgery, resulting in less peri and post operative suppression of the immune system. The tumour cell lines used in these studies were weakly immunogenic and reported to undergo spontaneous regression in 20 % of animals during the time they were observed for metastases. However, the potential for cancer cells to be distributed within the peritoneal cavity by the insufflating gas to cause wound metastases was not tested, because the tumour cells were injected only intradermally. Port site metastases may be caused by local, rather than general factors.

Bouvy *et al* (1996a) using an immunocompetent rat model, studied the effects of weight gain and tumour take, following open and laparoscopic resection of small bowel. They used a carcinogen induced colonic carcinoma cell line, which is weakly antigenic. The findings from this study showed there was a significant increase in the tumour take on the peritoneum, incision and bowel anastomosis in the group that underwent an open bowel resection, when compared to the group that underwent laparoscopic bowel resection. Their conclusion from this study was that laparoscopic surgery was associated with reduced tumour growth.

However, when they compared results with their control groups, one of which had only anaesthesia after the intraperitoneal injection of the tumour and the other, which had only CO_2 insufflation after the instillation of the tumour, they found that the tumour growth occurred significantly more in the CO_2 group. They concluded that CO_2 had a stimulatory effect on the growth of tumour. The findings from this study are important in the sense that they indicate that CO_2 enhances tumour growth in the peritoneal cavity. The mechanism of CO_2 enhancement of tumour growth is unclear. The other finding was that the surgical procedure itself may cause alterations to immunity.

Findings of increased tumour growth in the open small bowel resection group compared to the laparoscopic group may be a reflection of the effects caused by the magnitude of the surgical insult on the immune status of the animal and not the laparoscopic environment alone. The cells were injected into the peritoneal cavity at the end of the insufflation and laparotomy, hence essentially the effect of post operative stress and immune changes were tested, rather than the direct effect of laparoscopy.

Bouvy *et al* (1996b) reported another study, using the same model. They studied two experimental groups of rats, with controls in each group. In the first experimental group, they injected colon carcinoma cells 2 days before the rats underwent small bowel resection either laparoscopically or by open surgery and one group had only anaesthesia and served as a control group. In the second set of experiments, the rats underwent implantation of a piece of solid tumour in the sub-renal capsule, through a midline laparotomy, which was then closed. Two days later, the rats underwent a small bowel resection, either by laparoscopy, gasless laparoscopy or by the open technique. They also included a group of rats which served as controls. The findings from both of these experiments was that laparoscopy was associated with less peritoneal growth and comparing the laparoscopy groups, the tumour growth was less in the gasless laparoscopy group. It was concluded that laparoscopy was immunologically beneficial to the animal when compared to laparotomy and that gasless laparoscopy resulted in less tumour growth when compared to laparoscopy with CO_2.

In the first experiment, the laparoscopy was performed 2 days after the instillation of tumour cells and there may be some debate about the state of the cells at that time. It is possible that the peritoneal defence mechanisms would have already come into play and viable cells may have already implanted. There was the added trauma of bowel resection, apart from the difference in the presence or absence of CO_2 pneumoperitoneum.

In the second set of experiments, the animals underwent surgery to implant the cells on day 1 and two days later they had the additional surgical insult of a small bowel resection through a laparotomy, or laparoscopy with CO_2. This would amount to a significant degree of trauma and may be the cause for considerable metabolic and immunologic stress in the animal. This study demonstrates that alterations in immunity can cause a difference in tumour growth post operatively. It is difficult to assume these changes are due to the direct effect of CO_2, or the effects of insufflation.

Intraperitoneal growth and distribution of tumour cells following CO_2 laparoscopy

In a series of studies reported by Bouvy *et al* (1996b), they used the same animal model, but a modified experimental protocol. In each experiment, laparotomy, laparoscopy with CO_2 insufflation, or laparoscopy by elevation of the abdominal wall (the gasless technique) was used. In the first experimental group, they used a solid tumour harvested from a carrier rat. This solid tumour was introduced into the peritoneal cavity through a left upper quadrant incision and placed in the peritoneal cavity in the laparoscopy group. This was performed through a midline incision in the laparotomy group. In the laparoscopy group, trocars were introduced into the abdominal cavity. After 20 minutes of insufflation, the trocars were withdrawn before the extraction of the tumour in the laparoscopy group. The tumour was extracted through the midline in the laparotomy group. In the second experiment of this study, free tumour cells were injected into the peritoneal cavity before each procedure i.e. laparotomy, laparoscopy with or without CO_2.

The findings from the solid tumour model showed there was diffuse and extensive tumour growth. However, the peritoneal tumour load was significantly less in the gasless laparoscopy group, when compared to the CO_2 group.

In the free cell experiment, the CO_2 group showed significantly greater tumour deposits at the trocar sites and tumour growth in the peritoneum, when compared to the

gasless laparoscopy group. These studies demonstrate mechanisms which may be involved in tumour spread. The mechanism of abdominal wall metastasis may involve direct implantation at trocar sites, when contaminated trocars are withdrawn or when tumour specimen is withdrawn. The other possible mechanism being postulated by Bouvy *et al* (1996b), is that insufflation of the peritoneal cavity causes turbulence. At the port site, a concentration of tumour cells occurs, combined with gas leakage along side trocars causing a high local gas flow, they and others have termed this observation as the "chimney effect" (Hubens *et al,* 1996).

Volz *et al* (1996), in their study used nude mice, where two different cell lines from human ovarian carcinoma were injected intra peritoneally. Pneumoperitoneum was established and a group which underwent only anaesthesia served as a control group. The findings from this study showed that CO_2 pneumoperitoneum resulted in a significant increase in the seeding, number and size of intra-abdominal metastases. The findings from this study are important in that the effect of CO_2 alone on tumour cell growth without the interplay of immune factors was examined. However, immune factors may be necessary in preventing this phenomenon and this study does not address that factor.

Hubens *et al* (1996) in their study using an immunocompetent rat model, studied rats which underwent laparotomy or CO_2 pneumoperitoneum, with or without IV cannulae, to simulate ports introduced into the abdomen. Colon cancer cells were introduced intraperitoneally and CO_2 pneumoperitoneum maintained for 30 minutes. The findings from this study showed that the creation of a pneumoperitoneum did not enhance the implantation of these cells, but when ports were introduced, tumour growth occurred at the port sites. These metastases were situated in the subcutaneous tissue around the port sites, indicating that the mechanism may be direct contamination or due to gas leaks around the port sites.

Goldstein *et al* (1993) used a rat bladder carcinoma cell line and studied the pattern of implantation of tumour cells in the presence of pneumoperitoneum and peritoneal disruption. They also instilled heparin intraperitoneally, to study if it prevents the implantation of tumour. The findings from their study showed that the group which underwent mechanical peritoneal disruption, developed tumours more frequently and tended to have more tumours at the site of peritoneal disruption. Significantly smaller tumour volumes were noted in the group that received heparin. This study demonstrated that tumour cells implant in sites of tissue injury preferentially. Agents like heparin, which prevent cell adhesion, may be capable of preventing this.

Jacobi *et al* (1997a) using a colon carcinoma cell line evaluated the effect of laparoscopy with CO_2 and air on intraperitoneal and subcutaneous cell growth. They found that intraperitoneal growth of tumour occurred more after laparoscopy with air and subcutaneous growth of tumour occurred more with CO_2, when compared to controls.

Hamster model

Jones *et al* (1995) used a hamster model, where they injected a human colon cancer cell line during laparotomy or laparoscopy with ports. The findings from their study showed that the addition of low pressure and short duration of pneumoperitoneum, increased the rate of implantation of tumour cells in the incision and trocar puncture sites. This study also demonstrated that the presence of CO_2 enhanced tumour implantation and growth.

Studies on cell movement

Whelan *et al* (1996) used a rat model to investigate if the phenomenon of port site metastases is due to aerosolisation, caused by the insufflated CO_2. They instilled a melanoma cell line into rats, which underwent a static pneumoperitoneum or a continuous pneumoperitoneum lasting 10 minutes. The vented gas from the animal was passed through a culture media and growth in the media observed. Findings from their

study showed no evidence of aerosolisation, as there was no tumour growth in any of the media. However, the tumour inoculation was small and the duration of pneumoperitoneum very brief. The process of aerosolisation may depend on the total volume or quantity of cells within the cavity and also the rate and pressure flow of gas.

Studies on large animal models

Studies on intraperitoneal cell movement

Studies on large animals, such as the pig, have been used mainly to study intraperitoneal cell movement and physiological responses to laparoscopy. Hewett *et al* (1996) used the pig model to study the movement of human colon cancer cell within the peritoneal cavity. They evaluated whether CO_2 expelled from the peritoneal cavity and whether laparoscopic ports and instruments were contaminated with malignant cells. The findings from this study showed that there were more cells detected on the laparoscopic instruments and ports when compared to the venting gas. This study demonstrated a mechanism by which laparoscopic instruments and ports can contaminate wounds, when cells are deposited on them. This study does not explain how the cells are deposited on the ports and instruments. It is also uncertain if these redistributed cells, would eventually implant and develop into metastases.

Texler *et al* (1997) used the same animal model to compare the effect of dry CO_2 and humidified CO_2. They studied the distribution pattern of colon cancer cells retrieved from instruments and filters on venting cannula, following establishment of pneumoperitoneum and instrument introduction into the peritoneal cavity. They concluded that cells were aerosolised during CO_2 insufflation.

Knolmayer *et al* (1996) also used a porcine model and a similar protocol, but bubbled the escaping gas from the venting cannula through saline and found epithelial cells were recovered from the saline.

Allardyce *et al* (1996) used a porcine model, to study the effect of laparoscopy on the distribution of cells within the peritoneal cavity. They used He La cells labelled with isotopic Chromium for this study. The cells were introduced intraperitoneally and then the pigs underwent laparoscopic or open colectomies. The findings from this study suggested that the tumour cells were distributed throughout the peritoneal cavity. Ports used by the surgeon contained more cells, than the other ports. This finding may indicate that movement or activity and consequential leakage during insufflation may be a major factor. In this study only enumeration of cells and their distribution could be studied. This may not indicate the development of metastases, as this may be dependent on shed cell behaviour and the metastatic potential of the tumour cell line involved.

In vitro / in vivo studies on tissue cultures

Nduka *et al* (1997) demonstrated that an injection of rat colon cancer cells (after *in vitro* exposure to CO_2) into the rat's peritoneal cavity, resulted in a significantly enhanced tumour growth both intraperitoneally and at the wound.

Jacobi *et al* (1997a) studied *in vitro* cell growth of colon adenocarcinoma cell lines in the presence of air or CO_2. They found that both CO_2 and air caused a significant increase in the tumour cell growth.

Implications of findings from this work

Findings from the series of studies presented in this thesis have important clinical implications:

(a) With a significant increase in the development of wound recurrence following laparoscopic procedures where free malignant cells are likely to be present or liberated, port site recurrences are likely to be a clinically relevant problem.

(b) The presence of CO_2 pneumoperitoneum plays an important part in the development of port site metastases during laparoscopic manipulation. The use of a gasless technique or other gases may reduce the incidence to the same level as that in open surgery.

(c) CO_2 insufflation increased the distribution of cells and also enhanced tumour growth intraperitoneally. Thus CO_2 may have a direct effect facilitating tumour growth.

(d) The circulating CO_2 pneumoperitoneum is capable of transporting free cells during high pressure leaks, in the presence of adequate quantity of free cells. This combination of circumstances is likely to exist in the clinical situation, especially during desufflation and accidental slipping out of ports, creating an environment for the development of port site metastases.

(e) The alterations in immunity induced by the tumour bearing state influences the spread of tumour within the peritoneal cavity.

(f) The alterations to macrophage activation by CO_2 insufflation results in decreased production of TNF-α, when compared to laparotomy and gasless laparoscopy.

(g) The immune response in the small laparoscopic wound is decreased when compared to the larger incisions used for laparotomy, as evidenced by a decreased macrophage response. This attenuated immune response may facilitate the

implantation of tumour cells deposited at these wounds and result in port site metastases.

(h) CO_2 insufflation causes significant acidosis of the peritoneal surface, which may cause alterations in cellular integrity and immune response of the peritoneal cavity to tumour cells. A similar change is likely to occur in the clinical situation.

The mechanism of port site metastasis is multifactorial and further studies are mandatory to understand the precise mechanisms for the development of this phenomenon.

Future directions

1. There is evidence from these studies that CO_2 is capable of redistributing cells at moderate flow rates and transporting cells at high flow rates. This may be dependent on the amount of gas circulating. Further studies are necessary to clarify the relationships of the flow rates and insufflation pressures, with cell migration and distribution.

2. There is evidence to indicate that CO_2 causes increased growth of tumour cells in the peritoneal cavity. The mechanism is not clear. Further studies are necessary to study the possible influence of CO_2 on the micro-environment of tumour cells.

3. There is evidence to indicate that CO_2 causes metabolic changes on the peritoneal surface, one of which is a change in pH. Further studies are needed to investigate the peritoneal responses to this metabolic change and any influence on the pattern and growth of intraperitoneal tumours. The effect of inert gases which are biologically inactive like Helium, on port site and tumour growth should be investigated.

4. The presence of adequate viable free cells in the peritoneal cavity is necessary for the development of port site metastases. Studies to investigate the efficacy of strategies to kill free intraperitoneal tumour cells before they implant, should be undertaken. The effect of the postulated strategies remains to be investigated under laparoscopic conditions.

Possible strategies to prevent port site metastases

There are many strategies which have been advocated to prevent or avoid this problem. There is strong evidence that the presence of CO_2 pneumoperitoneum plays an important role in the development and growth of tumours (Jones *et al,* 1995; Volz *et al,* 1996). Hence, the use of gasless laparoscopy wherever feasible, may avoid the harmful effects of CO_2 pneumoperitoneum (Bouvy *et al,* 1996b). Gasless laparoscopy,

which uses a working space in the abdominal cavity without the use of pneumoperitoneum, is becoming more commonly used. With the development of better instruments to create this working space, this technique should become increasingly applicable. If pneumoperitoneum is necessary in situations for better visibility and space for operative manoeuvrability, then the use of inert gases, like Helium, which may cause the least metabolic disturbance, should be explored.

The use of non porous retrieval bags to remove malignant specimens have been advocated. However, this may only avoid implantation of tumour directly from the specimen to the port through which it is removed, but may not prevent implantation of free cells present in the peritoneal cavity to other port site wounds. Other preventative measures suggested include protection of the port site wound, either by mechanical or chemical means. The use of wound protectors, to protect wounds from coming into direct contact with the tumour specimen being extracted or tumour cells attached to the surface of the ports or instruments, is a logical measure to be tried (Copher *et al,* 1995; Sandor *et al,* 1995). These may also create an air tight seal and prevent gas leaks around the ports and this may prevent sudden desufflation.

One of the strategies suggested to prevent port site metastases is the instillation of intraperitoneal cytotoxic agents (Jacquet *et al,* 1996; Jacquet & Sugarbaker, 1996). Intraperitoneal instillation of chemotherapeutic agents have been found effective in the prevention of peritoneal dissemination in carcinoma of the stomach and ovarian malignancies (Sugarbaker *et al,* 1989). The instillation of heparin, to prevent cell adhesion, has also been found effective in experimental conditions (Goldstein *et al,* 1993).

The excision of laparoscopic port sites following procedures which involve malignancy has been proposed (Cotlar *et al,* 1996). The use of low-energy carbon dioxide laser light has been described for the 'sterilisation' of mastectomy fields, to prevent wound recurrence which may also be applicable to port site wounds

(Lanzafame *et al,* 1988). However, no experimental or clinical studies have been performed to assess if any of these measures are justified or practical in laparoscopic surgical situations.

Thus, there is growing evidence from clinical and experimental studies, that the incidence of wound metastasis following cancer surgery has increased by the use of laparoscopic techniques. This offsets the reported beneficial effect of laparoscopic surgery on systemic immunity. The reports of tumour implantation to port site wounds following laparoscopic surgery for early stage cancers and tumours of low malignant potential, suggests that this complication may have an adverse influence on survival and quality of life. The mechanisms which are involved in the development of this problem are not fully understood. The aetiologic factors are most likely to be multifactorial.

It is assumed that patients who undergo laparoscopic surgery have less pain, a shorter period of ileus and an early return to work. Even with all these reported benefits the important question is: Would the patient trade cure and long term survival, for short term recovery? There are serious doubts in the minds of surgeons who practice laparoscopic surgery for malignancy. As reported by Wexner, only 6% of the surgeons who advocate laparoscopic surgery for colonic malignancy, would have the same technology applied to themselves (Wexner *et al,* 1995). Evidence from studies on this experimental model indicates that port site metastases are likely to be a clinically relevant problem. A critical evaluation of the application of this technique in the surgical treatment of cancer should be undertaken before advocating its wide spread use. Until the issue of laparoscopy associated wound metastasis is fully understood, laparoscopic and thoracoscopic cancer surgery should be encouraged to take place only within the context of clinical trials.

Operations should not be trophies.
They must always be performed for
the greater good of the patient
- not for the glory or amusement of the surgeon.

Alexander J. Walt
Gerald Marks Lecture
S A G E S, Scientific Session - 1994

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APPENDIX

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