09M.D
E14

ASSESSMENT OF CD44 AND K19 AS MARKERS FOR CIRCULATING BREAST CANCER CELLS USING IMMUNOBEAD RT-PCR

A Thesis Submitted for the Degree of

Doctor of Medicine

by

Michael Campbell Eaton, MBBS, FRACS

Department of Surgery,

The University of Adelaide.

Submitted for degree in March, 1997.

TABLE OF CONTENTS

Abstract

Declaration

Acknowledgments

Table of Figures

Table of Tables

Table of Abbreviations

page

Chapter One: Introduction and Review of Literature 1-50

1.1 Breast cancer 1

1.2 Previous methods to detect circulating tumour cells 6

1.3 Current methods to detect circulating tumour cells 7

1.3.1 Immunocytochemistry 8

1.3.2 The polymerase chain reaction 13

1.3.3 Reverse transcription-polymerase chain reaction 15

1.3.4 Flow cytometry 20

1.3.5 Immunomagnetic cell isolation techniques 21

1.3.6 Clonogenic assay 21

1.4 Clinical results of detection of circulating tumour cells 22

1.4.1 Bone marrow 22

1.4.2 Peripheral blood 24

1.4.3 Peripheral blood stem cell collections 25

1.5 The development of immunobead RT-PCR 30

1.5.1 Selection of antibodies 30

1.5.2 Methods of identification of tumour cells isolated by immunobeads 34

1.5.3 CD44 35
1.5.3.1 Introduction 35
1.5.3.2 Definition, structure and function 36
1.5.3.3 Expression of different CD44 isoforms may confer metastatic potential 37
1.5.3.4 Distinction of benign from malignant tissues on the basis of CD44 variant expression 38
1.5.3.5 A proposed RT-PCR method for detection of circulating tumour cells based on abnormal CD44 expression 39
1.5.3.6 CD44 expression in tissues and cells 40
1.5.3.7 Prospects for the investigation of CD44 expression as a potential marker for breast cancer cells using immunobead RT-PCR 42
1.5.4 Cytokeratins 43
1.5.4.1 Introduction 43
1.5.4.2 Expression of cytokeratins in breast epithelium 44
1.5.4.3 K19 expression in haemopoietic cells 45
1.6 Summary 48

Chapter Two: General Materials and Methods 51-71

2.1 Reagents and solutions 51
2.1.1 Solutions used for cell culture 51
2.1.2 Solutions for molecular biology 52
2.2 Cell culture and cell manipulation 55
2.2.1 Cell lines and culture 55
2.2.2 Manipulation of cell lines from culture 55

2.2.3 Isolation of mononuclear cells from whole blood or bone marrow 56
2.2.4 Freezing cell lines and human samples 56
2.2.5 Recovering cell samples stored in liquid nitrogen 57
2.2.6 Cell counting and serial dilutions 57
2.2.7 Aliquots of cell lines were stored at -80°C for use as positive controls for RT-PCR 58
2.2.8 Micropipette aspiration of cells for RT-PCR 58
2.3 Collection of human samples for development of the immunobead RT-PCR method 59
2.3.1 Breast cancer specimens 59
2.3.2 Axillary lymph nodes replaced by tumour 60
2.3.3 Normal lymph node 60
2.3.4 Bone marrow and peripheral blood samples 61
2.4 Molecular biology techniques 61
2.4.1 Preparation of total RNA 61
2.4.2 The polymerase chain reaction 62
2.4.3 Gel electrophoresis 63
2.4.4 Southern blot analysis 63
2.5 Summary of the immunobead RT-PCR method 65
2.5.1 Labelling immunobeads with Ber-EP4 65
2.5.2 The efficacy of labelling of beads 66
2.5.3 Incubating samples with the immunobeads 66
2.5.4 Application of the RT-PCR assay to the immunobead cell isolates 67
2.6 Collection of human samples for testing by immunobead RT-PCR 68
2.6.1 Bone marrow biopsies 68

2.6.2 Peripheral blood samples....69
2.6.3 Peripheral blood stem cell collections....69
2.6.4 Peripheral blood samples taken at the time of PBSC harvest....71

Chapter Three: Selection of an Antibody for Tumour Cell Isolation by Immunobeads 72-78

3.1 Introduction....72
3.2 Results of antibody binding to breast cancer cells....73
3.2.1 Flow cytometry....73
3.2.2 Immunohistochemistry....75
3.3 Discussion....75

Chapter Four: RT-PCR for Very Small Numbers of Cells 79-100

4.1 Introduction....79
4.2 RNA extraction....79
4.2.1 Introduction....79
4.2.2 Detergent lysis of cells by Nonidet P-40....80
4.2.3 Assessment of methods of cell lysis....82
4.2.3.1 Introduction....82
4.2.3.2 Comparing NP-40 with heat lysis....82
4.2.3.3 Comparing NP-40 with freeze thawing....83
4.3 Reverse transcription....84
4.3.1 Introduction....84
4.3.2 Reverse transcription from very small numbers of cells....84
4.3.3 RNasin/DTT is necessary for RT by cell lysis....89
4.4 Conclusions regarding the assay for reverse transcription....90
4.5 Polymerase chain reaction....91

4.5.1 Introduction 91
4.5.2 The optimum volume of reverse transcription product to add to the PCR mix 92
4.5.3 The amount of PCR product loaded for gel electrophoresis 95
4.5.4 Other aspects of PCR 97
4.6 Summary 100

Chapter Five: Assessment of CD44 for Immunobead RT-PCR 101-112
5.1 Introduction 101
5.2 Materials and methods 102
5.2.1 PCR Primers for CD44 102
5.2.2 PCR Conditions for CD44 103
5.3 Results 104
5.3.1 CD44 expression by haemopoietic cells 104
5.3.2 CD44 expression by breast cancer cell lines 105
5.3.2.1 CD44 expression from low cell numbers prepares by serial dilution 105
5.3.2.2 CD44 expression from very small numbers of cells selected by micropipette 107
5.3.3 CD44 expression by metastatic human breast cancer cells 108
5.3.4 Immunobead RT-PCR for CD44 using whole blood only 111

Chapter Six: Expression of K19 by RT-PCR 113-124
6.1 Introduction 113
6.2 Conditions for exclusion of pseudogene amplification 115
6.3 Sensitivity of K19 RT-PCR 116
6.4 Expression of K19 by haemopoietic cells 118

6.5 Discussion 120
6.5.1 Design of primers for K19 PCR 120
6.5.2 Choice of PCR annealing temperature for exclusions of pseudogene amplification 121
6.5.3 Sensitivity of K19 RT-PCR 122
6.5.4 Specificity of K19 RT-PCR 123

Chapter Seven: Technical Aspects of Immunobead RT-PCR 125-134

7.1 Introduction 125
7.2 The optimum number of immunobeads per sample 125
7.3 Sensitivity of Immunobead RT-PCR 126
7.4 Technical difficulties of working with archival specimens 129
7.5 Denaturation of RNA can be avoided for Immunobead RT-PCR 131
7.6 Positive controls for Immunobead RT-PCR 133
7.7 Summary 134

Chapter Eight: Patient Results 135-150

8.1 Bone marrow biopsies 135
8.2 Peripheral blood stem cell harvests 140
8.3 Peripheral blood samples 141
8.4 Peripheral blood samples taken at the time of stem cell harvest 142
8.5 Are circulating carcinoma cells separated from mononuclear and stem cells during leukapheresis? 146

Chapter Nine: Caveats 151-165

9.1 Introduction 151
9.2 Some populations of haemopoietic cells can express K19mRNA 155

9.3 PMA activates normal MNCs to express K19.....156
9.4 CD44 PCR to test patient negatives and samples 159

Chapter Ten: Summary, Conclusions and Further Research 166-173

10.1 Introduction 166
10.2 Immunomagnetic cell isolation can be applied to breast cancer cells.....167
10.3 CD44 expression by RT-PCR cannot differentiate carcinoma cells from haemopoietic cells.....168
10.4 Results of cytokeratin 19 as a marker for carcinoma cells using Immunobead RT-PCR.....169
10.5 Limitations of the clinical results using K19 171
10.6 Further research 172

Appendix

Bibliography

ABSTRACT

PURPOSE:

Detection of occult metastases and circulating tumour cells in patients with breast cancer may prove useful in determining prognosis and in planning and monitoring systemic therapies. The identification of carcinoma cells in peripheral blood stem cell (PBSC) harvests may predict relapse after high dose chemotherapy with PBSC transplantation.

This work presents the development of an assay for reverse transcription-polymerase chain reaction (RT-PCR) to be applied to the immunomagnetic isolation of carcinoma cells, as a possible means of detecting small numbers of breast cancer cells in a haemopoietic environment. The messenger RNA expression of two different genes, CD44 and the cytokeratin K19, was assessed for suitability as tumour markers for the Immunobead RT-PCR method, and clinical results using K19 are presented.

METHODS AND RESULTS:

An assay for RT-PCR from very small numbers of cells was developed, using detergent lysis of cells for RNA extraction. Taking fresh tumour cells from a lymph node, it was demonstrated that single metastatic breast cancer cells could express more than one isoform of CD44. CD44 did not prove useful as a tumour marker for Immunobead RT-PCR in a haemopoietic environment, because the CD44 isoform expression of haemopoietic cells could not be distinguished from that of carcinoma cells. The expression of K19 was shown to be specific for epithelial cells, but occasionally K19 transcripts could be amplified from haemopoietic cells of normal individuals. Immunobead RT-PCR using K19 expression as the tumour marker allowed detection of one carcinoma cell amongst one million leukocytes in peripheral blood.

Circulating tumour cells were not detected in any samples of peripheral blood taken at random from patients with advanced breast cancer, nor in any PBSC samples. K19 transcripts were amplified from four samples of peripheral blood taken from patients at the time of PBSC harvest.

CONCLUSIONS:

Immunobead RT-PCR was shown to be a highly sensitive method of detecting breast cancer cells in a haemopoietic environment. Results using K19 as the marker should be interpreted with caution. Results suggested that the mobilisation of stem cells is accompanied by mobilisation of breast cancer cells into peripheral blood, but that circulating tumour cells may be excluded from stem cell harvests.