REPERFUSION INJURY IN FOCAL CEREBRAL ISCHAEMIA

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Abstract

Reperfusion injury is a complex, multifactorial process which is of great importance in current clinical practice. Two aspects of reperfusion injury following focal cerebral ischaemia have been studied.

The aetiology of the "no-reflow" phenomenon has been shown to be primarily the adhesion of activated neutrophils to ischaemic endothelium. The first study undertaken compared relative microvascular patency in basal ganglia subjected to 3 hours ischaemia and 1 hour reperfusion with basal ganglia subjected to 4 hours ischaemia and no reperfusion. No significant difference in vessel patency was observed for each vessel size range between the two groups, suggesting that lengthy periods of focal ischaemia result in irreversible microvascular occlusions in the vascular bed, and thus shorter periods of ischaemia would be more appropriate time periods for studying this phenomenon.

The synthetic RGD peptide TP9201, which blocks integrin $\alpha IIb\beta 3$, underwent pharmacokinetic and pharmacodynamic studies in the primate model. The potent effect on platelet aggregation was confirmed by platelet aggregometry, and a moderate elevation in template bleeding time was also found. The short half-life of this agent suggests a potential clinical role in blocking platelet aggregation. However, at the dose used in this model of focal cerebral ischaemia and reperfusion, 3 of 4 animals infused with this agent sustained intracerebral haemorrhages into the reperfused basal ganglia. Microvascular patency was improved but this finding is likely to be artefactual.

The role of endothelium in the modulation of the coagulation system during cerebral reperfusion was studied using in-situ hybridisation. This technique uses antisense probes to bind to specific mRNA expressed in tissue sections. This permits the mRNA of interest to be localised at a cellular level and semi-quantitated. The technique was developed to the stage where subcloned probes for Tissue Factor, tissue-type Plasminogen Activator and von Willebrand Factor were prepared and used. Hybridisation was achieved, but non-specific binding proved to be a problem which was not able to be corrected by modification of the technique. With further development, this will be a powerful tool for studying gene expression in this model.

Statement of Originality

This work contains no material which has been accepted for the award of any other degree or diploma in any university or any other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except when due reference has been 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.

Robert A. Fitridge

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Abbreviations

DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DTT	dithiothreitol
EDRF	endothelium-derived relaxing factor
EDTA	ethylene diamine tetra-acetic acid
FGF	Fibroblast growth factor
GMP	guanosine 5'-monophosphate
HUVEC	human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule - 1
IL-1	Interleukin-1
IPTG	isopropyl-β-D-thiogalactopyranoside
I/R	ischaemia/ reperfusion
ISH	in-situ hybridisation
LPS	lipopolysaccharide
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
MCAO/R	middle cerebral artery occlusion / reperfusion
NO	nitric oxide
NOS	nitric oxide synthetase
OCT	Tissue Tek® OCT embedding compound [10% polyvinyl alcohol, 4% polyethylene glycol, 86% non-reactive ingredients]
PAF	Platelet activating factor
PAI-1	type-1 plasminogen activator inhibitor
PBS	phosphate-buffered saline
PMN	polymorphonuclear leukocyte
RGD	Arginine-Glycine-Aspartate
RNA	ribonucleic acid
RNAse	ribonuclease
SDS	sodium dodecyl sulphate
sLe^x	sialyl $Lewis^x$
SOD	superoxide dismutase
TF	Tissue factor
TGF	Transforming growth factor
TNF	Tumour necrosis factor
tPA	tissue-type Plasminogen activator
Tris	Tris (hydroxymethyl) aminomethane
XGal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1

Reperfusion injury background

The extent of tissue damage which occurs as a result of ischaemia is dependent upon several factors: the duration of ischaemia, the level of blood flow during ischaemia and the sensitivity of the tissues to ischaemia. Upon restoration of tissue perfusion, oxygen and metabolic substrates are returned to tissues which have been reversibly damaged. However, complex interactions take place between the blood elements and vessel endothelium, potentially resulting in an exacerbation of tissue damage. This deleterious effect of return of blood flow to tissues is termed "reperfusion injury". Reperfusion injury implies that reperfusion with "unmodified" blood is not an ideal perfusate for ischaemic tissues (Hallenbeck and Dutka, 1990).

Reperfusion injury has been studied most extensively in the myocardium. In 1960 , Jennings et al suggested that reperfusion may accelerate the development of necrosis in irreversibly injured myocytes.

Ames and coworkers (1968) showed that following relatively short periods of ischaemia, localised areas of the brain failed to reperfuse on restoration of blood flow. They demonstrated this by showing decreased carbon perfusion, and described this finding as the "no-reflow phenomenon". Ames suggested that increased blood viscosity and reduction of capillary lumen secondary to perivascular oedema were the most likely causes of this condition. The "no-reflow phenomenon" is a major focus of the experimental work undertaken in this laboratory and in this thesis.

The concept that inflammation is a major mediator of tissue injury after ischaemia has been confirmed by many studies in which anti-inflammatory agents, complement depletion (Crawford et al, 1988), anti-neutrophil strategies (Engler et al, 1986), and administration of prostacyclin analogues (Simpson et al, 1987) have been shown to reduce infarct size.

This background discussion of reperfusion injury will cover the role of the major mediators of this complex pathological process. Many of the studies discussed were performed in organ systems other than brain, and are included as the basic mechanisms appear to be very similar in each organ system. Much of the available data describing endothelial behaviour and the

role of cell adhesion molecules in response to ischaemia/reperfusion and inflammation have been obtained in cell culture experiments.

The roles of cell adhesion molecules and endothelial modulation of coagulation in reperfusion injury are highlighted because of their relevance to the experimental work undertaken during this research period.

1.1 Pathophysiology of reperfusion injury in brain following focal cerebral ischaemia

Vascular endothelium plays a variety of crucial roles in response to physiological and pathological stimuli. Endothelium is an actively anticoagulant and antithrombotic surface, maintains cerebral vascular tone by producing several vasoactive factors, and mediates inflammatory and immune responses.

Vascular endothelial dysfunction is a critical early phenomenon in all forms of ischaemia/reperfusion (I/R). Dysfunction occurs within several minutes of hypoxia in the cerebral circulation and reperfusion leads to major alterations in procoagulant activity, vascular tone and changes in endothelial adhesion.

1.1.1 Oxygen-derived free radicals

A free radical is a molecule that contains an unpaired electron. These reactive oxygen species are unstable and hence highly reactive. Generated from the reduction of molecular oxygen, they include hydrogen peroxide (H_2O_2), hydroxyl radical (°OH), superoxide anion radical ($O_2^{-\circ}$) and the perhydroxyl radical ($HO_2\circ$). They can act as oxidants or reductants.

Free radicals may be generated by mitochondria, activated leukocytes (Britigan et al, 1986), from endothelial cells following ischaemia/ reperfusion (Jarasch et al, 1986), from tissues containing activated leukocytes during inflammation (Petrone et al, 1980) and enzymes such as cyclo-oxygenases or xanthene dehydrogenase (McCord, 1985).

Lipid peroxidation at the cellular membrane level represents a free radical-mediated chain reaction that alters or destroys membrane phospholipids. Transition metal ions, particularly Fe^{++} are involved in these redox reactions (Braugher and Hall, 1989).

Reperfusion is accompanied by the generation of free radicals and other reactive species which appear to be responsible for tissue damage, rather than tissue acidosis, which may of itself have a cytoprotective effect in hypoperfusion states (Kitakaze et al, 1988). Since reperfusion injury requires the re-establishment of flow and delivery of oxygen, free radicals are likely to play a major role in early endothelial dysfunction in reperfusion injury and that damage may be prevented by controlling these radicals. Studies of isolated hearts have confirmed that free radicals are generated during reperfusion and that cardiac dysfunction following reperfusion was proportional to the free radical concentration in the perfusate leaving the heart (Arroyo et al, 1987, Zweier, 1988). Thus oxygen toxicity is a significant factor in damage previously attributed to lack of oxygen (Levine, 1993).

Generation of free radicals within the microenvironment formed between activated polymorphs and activated endothelial cells leads to increased vascular permeability and tissue damage. Reperfusion enhances free radical generation by recruiting leukocytes to the reperfused tissue (Lucchesi et al, 1989).

Injury caused by free radicals can be limited by endogenous superoxide dismutase (SOD), glutathione peroxidase and catalase (Roos et al, 1980, Barsacchi et al, 1984). Studies have also shown a reduction in free radical generation by the addition of SOD and catalase (Grammas et al, 1993).

1.1.2 Calcium

Extracellular calcium concentration in areas of injured brain falls from 1 mMol to less than 0.1 mMol rapidly following injury as calcium moves into ischaemic cells which have lost their capacity to maintain calcium homeostasis (Young, 1986). Calcium concentration in the extracellular space then rises over a period of hours due to diffusion of calcium ions from surrounding tissues. This exposes surviving neurones, which initially were able to maintain their calcium homeostasis, to a calcium load. "Delayed acceleration" of tissue damage appears to occur as extracellular calcium levels return to normal in models of ischaemic neuronal injury.

Ischaemia can also disrupt calcium homeostasis by interfering with sodium-potassium ATPase function, resulting in cell membrane depolarisation and intracellular sodium

accumulation. This can allow calcium to enter cells as sodium is pumped out. In addition, sequestering mechanisms become impaired within cells, resulting in calcium movement from the endoplasmic reticulum into the cytosol. This can result in activation of proteases and phospholipases (potentially leading to generation of free radicals), generation of arachidonic acid metabolites and membrane damage. Reperfusion can also result in an acceleration of calcium influx into cells by injuring cell membranes and hence increasing permeability (Murphy et al, 1987). The exact role of cytosolic calcium accumulation in the evolution of neuronal and myocardial injury after ischaemia remains unclear (Cheung et el, 1986; Uematsu et al, 1989).

1.1.3. Vessel tone

Vessel tone is influenced by the endothelium. In 1980, Furchgott and Zawadski demonstrated that endothelial-dependent relaxation of vessels is mediated via endothelium-derived relaxing factor (EDRF), which was later shown to be nitric oxide (NO). Nitric oxide is generated by nitric oxide synthetase (NOS), which occurs widely in the CNS and may be activated by calcium influx in reperfusion injury (Furchgott and Zawadski, 1980). Nitric Oxide Synthetase is localised in the neurones and vascular endothelium (Ross et al, 1990) and will generate NO from L-arginine. After being released by endothelium, NO stimulates soluble guanyl cyclase in smooth muscle, resulting in increased cGMP and hence relaxation. This is similar to the effect of nitrate vasodilators (Faraci, 1992). In addition to being a vasodilator, NO may also influence vascular tone by regulating production of endothelin (ET-1) and platelet-derived growth factor β (PDGFβ) (Kourembanas et al, 1993).

Nitric Oxide also inhibits platelet aggregation by a cyclic GMP-dependent mechanism and acts synergistically with prostacyclin in this role by increasing platelet concentration of cyclic AMP (Moncada and Higgs, 1993). Unlike prostacyclin, NO also inhibits platelet adhesion.

Nitric oxide can rapidly react with superoxide radicals to form the peroxynitrite anion ($ONOO^-$) which decomposes when protonated to the potent oxidants, hydroxyl radical and nitrogen oxide (Blough and Zafiriou, 1985). Superoxide dismutase may prevent NO decomposition by superoxide radicals, which would help maintain normal vasodilatation and

block thrombosis. SOD can also inhibit the formation of peroxynitrite which could otherwise decompose to form cytotoxic reactive oxygen species.

Vascular endothelium also produces several vasoconstrictor substances. These include endothelin (ET-1), which has been shown to be released in vitro in response to thrombin, adrenalin and calcium ionophore A23187 (Yanagisawa et al, 1988).

Endothelin is a 21-amino acid peptide. Three forms have been isolated, with only ET-1 being produced by human endothelial cells . ET-1 is the most potent vasoconstrictor known and is believed to play a role in cardiovascular homeostasis and possibly pathological states such as hypertension, atherosclerosis and reperfusion injury (Änggård et al, 1990)

1.1.4 Excitotoxins

The excitatory amino acids, glutamate and aspartate, are released during ischaemia and the toxic effect of glutamate exacerbates ischaemic neuronal injury. These excitotoxins appear to act via activation of the N-methyl-D-aspartate (NMDA) receptor, resulting in accumulation of calcium in the cytosol (Rothman and Olney, 1986; Novelli et al, 1988), the effects of which have been discussed above.

1.1.5 "No-reflow" phenomenon

Following relatively brief periods of occlusion of the artery supplying a tissue bed, a significant proportion of microvessels fail to reperfuse upon restoration of blood flow. The mechanisms of microvascular occlusions include:

(1) leukocyte plugging of microvessels (Schmid-Schonbein, 1987; del Zoppo et al, 1991);

(2) fibrin deposition in microvessels (Okada et al, 1994);

(3) endothelium becoming procoagulant (Ogawa et al, 1991).

Other potential contributors include endothelial cell swelling (Leaf, 1973), endothelial luminal membrane tags (Dietrich et al, 1987) and perivascular oedema (Fischer et al, 1977).

1.1.6 Role of leukocytes in reperfusion injury

Leukocytes are stiff, visco-elastic cells which are slightly larger than capillaries and must deform to pass along capillaries. Leukocytes appear to contribute to the injury initiated by arterial obstruction. Capillary plugging by granulocytes has been shown to be the mechanism of microvascular occlusion occurring in the no-reflow phenomenon in heart and brain (Engler et al, 1986; del Zoppo et al, 1991; Garcia et al, 1994). One adherent leukocyte, trailed by a rouleau of packed red blood cells appears to occlude each capillary. Obstruction of several adjacent capillaries may result in underperfusion of individual cells and thus a local hypoxic environment.

In a recent study of brain infarction in rats, leukocytes (particularly polymorphonuclear [PMN]) were detected in the capillaries and venules of the ischaemic region as early as 30 minutes after arterial occlusion. The percentage of occluded microvessels increased from 8.5% at 30 minutes of ischaemia to 50.5% at 4 hours of ischaemia. The number of intravascular granulocytes peaked at 12 hours after MCA occlusion (seen to be occluding 10.6% of microvessels). However few intravascular PMNs were seen after 72 hours . The number of parenchymal granulocytes peaked at 24 hours, denoting the time-course of parenchymal infiltration of leukocytes in cerebral infarction (Garcia et al, 1994).

Leukocytes contribute to the endothelial dysfunction which occurs in I/R by several mechanisms:

1. Adherent, activated leukocytes release reactive oxygen metabolites (particularly superoxide radicals) at the leukocyte-endothelial interface. These may inactivate EDRF as well as having direct toxic effects on endothelium.
2. Activated leukocytes can release a variety of cytokines including interleukin-1 (IL-1) and tumour necrosis factor (TNF). Tumour necrosis factor can inhibit NO release.
3. Activated leukocytes can damage endothelial cells and increase permeability by releasing other cytotoxic agents such as proteases, cationic proteins and collagenases (Ma et al, 1992).

Using a myocardial model, several studies have demonstrated a relationship between infarct size following reperfusion and the amount of leukocyte infiltration (Lucchesi et al,1989; Lucchesi and Mullane,1986; Romson et al, 1983). Engler et al. (1986) demonstrated

significant amelioration of "no reflow" and tissue damage in myocardium by leukocyte depletion.

The role of activated leukocytes on infarct size in the cerebral model has not been as thoroughly studied as in the heart. However Hallenbeck et al. (1986) demonstrated accumulation of indium-labelled leukocytes in regions of ischaemic brain during the first four hours post-ischaemia in an air embolism model, although this model cannot be used to extrapolate directly to the clinical setting. Bednar et al. (1991) demonstrated a significant reduction in infarct size in neutropaenic subjects in a rabbit model using autologous clot, whereas thrombocytopaenic animals showed no significant change.

In a rat model of middle cerebral artery occlusion, infiltration of leukocytes into the ischaemic tissue was noted. Significantly greater infiltration was noted with reperfusion, using a tissue myeloperoxidase activity assay. Thus cerebral reperfusion results in leukocyte recruitment. This study also demonstrated increased Leukotriene B4 (a chemotactic agent) receptor binding in a parallel manner, suggesting that binding occurs to receptors located on neutrophils (Barone et al, 1992).

In a recent study of human cerebral infarcts using technetium-99m hexamethylpropyleneamine oxime (^{99m}TcHMPAO)-labelled leukocyte brain single-photon emission computed tomography (SPECT), leukocyte infiltration was seen in areas of perfusion defects (scans performed within first week after infarction), persisting at least five weeks after onset and then declining (Wang et al, 1993).

Leukocyte depletion (Aspey et al, 1989) or leukocyte blockade using antibodies (Mori et al, 1992) have been shown to modify but not reverse the "no-reflow" phenomenon, suggesting that other factors play a significant role. No study has shown a significant, well-documented effect on neurological outcome by blockade of leukocyte function, although Vasthare et al (1990) found better preservation of cortical function in rats subjected to incomplete cerebral ischaemia (measured by somato-sensory evoked potential activity) in leukopaenic animals compared to those with normal leukocyte counts.

Adherence of leukocytes and monocytes to the endothelium of the post-capillary venules as well as recruitment of leukocytes into the adjacent tissue is mediated by several cell adhesion molecules; members of the integrin family, all 3 selectins and all members of the

immunoglobulin supergene family. Many of these cellular adhesion molecules are regulated by multiple, complex mechanisms in response to a variety of cytokines and inflammatory mediators.

These groups of cell adhesion molecules will be described separately and the mechanisms of leukocyte-endothelium adherence will be discussed.

1.1.7 Cell Adhesion Molecules and their role in Reperfusion Injury

(a) Selectins

Selectins are a family of membrane glycoproteins, each of which are involved in cell - cell adhesion events (Bevilacqua et al, 1991). All 3 human selectins comprise a NH2 - terminus formed by a 120 amino acid lectin domain. This domain is followed by a sequence of 35 - 40 amino acids similar to an epidermal growth factor (EGF) repeating structure . This single EGF element is followed by a variable number of repeating elements ("consensus repeats") which are each about 62 amino acids long and are termed "complement binding" (CB) elements . All selectins are anchored to the cell membrane by a single transmembrane region which is followed by a short 17-35 amino acid tail.

Different mechanisms regulate the presence of each of the 3 selectins at the cell surface (Vestweber, 1992).

P-selectin

P-selectin (GMP-140, PADGEM, CD62) is constitutively expressed in normal vessels, particularly post-capillary venules, where it is stored in the Weibel-Palade bodies of endothelial cells. It is also found in the α-granules of platelets.

Inflammatory or thrombotic mediators such as thrombin and histamine induce fusion of the Weibel-Palade bodies to the endothelium within minutes, resulting in neutrophil and monocyte binding (Sugama et al, 1992). The surface expression of P-selectin in endothelial cells is transient and returns to basal levels within 20-60 minutes as a result of endocytosis into new Weibel-Palade bodies (Hattori et al, 1989). It remains on the surface of platelets for at least 1 hour after in-vitro activation with thrombin (Geng et al, 1990).

Okada et al (1993) recently demonstrated sustained endothelial expression of P-selectin at 24 hours following focal cerebral ischaemia and reperfusion, and suggested that continued

generation of thrombin within the microvasculature secondary to exposure of circulating blood to subendothelial Tissue Factor (TF) may be the cause of this finding.

P-selectin is a receptor for neutrophils and monocytes but not lymphocytes. Expression of P-selectin on endothelium activated with thrombin or histamine suggests that this adhesion molecule plays a major role in mediating very rapid binding of neutrophils and monocytes to venules exposed to these agonists. Activated platelets expressing P-selectin may facilitate neutrophil and monocyte recruitment to sites of thrombosis or haemorrhage. At sites of inflammation without haemorrhage, emigrating leukocytes might recruit activated platelets into tissues by binding to P-selectin.

Circulating plasma P-selectin has been detected in normal humans in low concentrations (≈0.1-0.3 μg/ml) and has been shown to be anti-inflammatory by resulting in down-regulation of CD18 - dependent neutrophil adhesion and subsequent respiratory burst. Thus, it may be of importance in preventing inadvertent activation of circulating neutrophils (Dunlop et al, 1992).

The ligands for the selectins appear to be oligosaccharides that are recognised by the N-terminal lectin-like domains of the selectins (McEver, 1991). Recently, sialyl-Lewis X (sLe^x), a ligand for P-selectin and E-selectin was shown to dramatically reduce lung injury generated by Cobra Venom Factor (CVF), when infused into rats. This injury has been well-characterised as oxygen-derived free radical-mediated, and P-selectin and neutrophil-dependent (Mulligan et al, 1993).

Endothelial cells stimulated with thrombin or histamine also synthesise the phospholipid, platelet-activating factor (PAF). After synthesis, PAF is rapidly presented on the activated endothelial surface, where it can play a key role with P-selectin in PMN adhesion and activation (Zimmerman et al, 1990; Coughlan et al, 1994).

E-selectin

E-selectin is induced on vascular endothelial cells by cytokines such as interleukin-1 (IL-1), tumour necrosis factor-α (TNFα), lipopholysaccharide (LPS) and requires *de novo* mRNA and protein synthesis. E-selectin is expressed within 4 hours of exposure to agonists and is rapidly down-regulated, attaining basal levels within 16-24 hours. It is not present in normal endothelium but is found in post-capillary venules at sites of inflammation (Cotran et al, 1986). Sialyl $Lewis^x$ appears to be the ligand for this selectin. E-Selectin is a receptor for

neutrophils and monocytes but not lymphocytes,and has recently been shown to provide greater strength of rolling adhesions than P-selectin. Slower rolling may lead to a more localised neutrophil accumulation in adjacent tissues (Lawrence and Springer, 1993).

L-selectin

L-selectin is a calcium-dependent, lectin-like adhesion receptor which is constitutively expressed on the cell surface of lymphocytes, neutrophils and monocytes. Leukocyte L-selectin also contains sLe^x, the ligand for P- and E-selectin. Following activation by chemotactic factors (such as IL-1 and TNF) or phorbol esters, L-selectin is shed within minutes from the surface of neutrophils. The same stimuli cause an increase in expression of the Mac-1 (β2) integrin on the cell surface, suggesting that L-selectin may act before the β2 integrin in the process of adhesion. This is further supported by studies showing L-selectin antibodies inhibit "rolling" of leukocytes in-vivo (von Adrian et al, 1992), and that anti-β2 antibodies do not affect rolling but block subsequent firm adhesion of leukocytes to venular endothelium (von Adrian et al, 1991).

Lawrence and Springer (1991) demonstrated in-vitro that only P-selectin, but not ICAM-1 could support rolling of neutrophils under flow conditions on a lipid bilayer containing the glycoproteins. Thus rapid expression of L- and P-selectins are probably involved in very early (reversible) PMN binding while β2 integrins bind to up-regulated ICAM-1 at a later step (Sugama et al, 1992).

(b) Integrins

Integrins are heterodimer transmembrane glycoproteins. The α subunits vary in size between 120 and 180 kilodaltons and are each noncovalently bound to a β subunit (90-110 kdaltons). Most cells express several integrins. There are currently 14 known α and 8 β subunits (Hynes, 1992).

Both subunits of integrins are transmembrane glycoproteins with a single hydrophobic transmembrane segment. The cytoplasmic domains are short (≤ 50 amino acids) except for β4. The extracellular domains associate to form the αβ heterodimers.

The leukocyte-specific β2 integrins are still referred to by earlier names : αL β2 = LFA-1; α_M β2 = Mac-1.

Individual integrins can often bind to more than one ligand and individual ligands are more often than not recognised by more than one integrin. The majority of ligands are extracellular matrix proteins involved in cell-substratum adhesion.

Some ligands (such as fibrinogen) can also mediate cell-cell aggregation and some recognise membrane proteins of the immunoglobulin superfamily (ICAM-1, ICAM-2, VCAM-1) and mediate cell-cell adhesion (Hynes, 1992).

The first integrin binding site to be defined was the Arg-Gly-Asp (RGD) sequence which is present in fibronectin , vitronectin and several other adhesion proteins. This site is recognised by $\alpha_{IIb}\beta 3$, $\alpha 5\beta 1$ and probably all $\alpha_v\beta$ integrins . (vide infra page55). Different sequences are recognised by other integrins, eg. $\alpha 4\beta 1$ binds Glu-Ile-Leu-Asp-Val (EILDV) and $\alpha 2\beta 1$ binds Asp-Gly-Glu-Ala (DGEA) in type I collagen. $\alpha_{IIb}\ \beta 3$ also recognises the sequence Lys-Gln-Ala-Gly-Asp-Val (KQAGDV) in fibrinogen.

The specificity and affinity of a given integrin receptor on a given cell are not constant. On resting circulating platelets, integrin $\alpha_{IIb}\ \beta 3$ does not bind any of its soluble ligands. If this were not the case, widespread thrombosis would occur. Unactivated platelets bind to surface-bound fibrinogen via $\alpha_{IIb}\ \beta 3$ and become part of haemostatic events which have already commenced. However, only after agonist activation of platelets (eg. by thrombin, collagen) does $\alpha_{IIb}\ \beta 3$ become an effective receptor for fibrinogen or its other ligands (Kieffer & Phillips, 1990). Activation is associated with a conformational change in the $\alpha_{IIb}\ \beta 3$ receptor, and further change occurs with ligand binding.

Activation of leukocytes is required for expression of the ligand-binding activities of the $\beta 2$ integrins and results in conformational changes in the integrin.

In response to tissue injury, neutrophils and monocytes must attach to endothelium in order to leave the bloodstream and migrate to the injured tissues. These events involve several adhesion proteins, amongst which the $\beta 2$ integrin is central. The first event in this process is that leukocytes "roll" along the vessel wall. This is mediated by selectins. The integrins on these "rolling" leukocytes remain inactive until activated by inflammatory mediators (eg. complement C5a, tumour necrosis factor (TNF)).

(c) Intercellular adhesion molecules

Intercellular adhesion molecule -1. (ICAM-1).

As previously mentioned, leukocyte adhesion to the endothelium is primarily mediated by the leukocyte β2 integrin (CD18) binding to its endothelial ligand, intercellular adhesion molecule-1 (ICAM-1). ICAM-1 is a member of the immunoglobulin supergene family and contains 5 immunoglobulin domains (Springer, 1990). It is constitutively expressed at low levels on the surface of endothelial cells and is induced and markedly up-regulated by cytokine stimulation. This process requires RNA transcription and ICAM-1 expression is maximal at 9-24 hours. The pro-inflammatory cytokines TNF-α, IL-1, interferon-γ and lipopolysaccharide (LPS) have been shown to up-regulate ICAM-1 expression in endothelial cells (Pober, 1988; Munro et al, 1989; Wong and Dorovini-Zis, 1992). The ligand for ICAM-1 is the β-2 integrin, LFA-1, which is expressed by neutrophils, lymphocytes and macrophages.

Using cultured brain microvascular endothelial cells, up to 20 hours of hypoxia did not lead to ICAM-1 mRNA up-regulation, however up-regulation was noted at 4, 12 and 24 hours of reoxygenation (Hess et al, 1994). This effect was significantly reduced by N-acetyl-L-cysteine. Thus free radical generation appears to play a major role in ICAM-1 upregulation in reperfusion.

Two recent studies have shown that ICAM-1 expression is enhanced during myocardial (Yamazaki et al, 1993) and cerebral (Okada et al, 1993) ischaemia/reperfusion. Furthermore, two studies (Ma et al, 1992, Yamazaki et al, 1993) demonstrated decreased infarct size and neutrophil infiltration in animals treated with antibodies to ICAM-1.

A second endothelial adhesion molecule, ICAM-2 also binds the $\alpha_L\beta_2$ integrin and is constitutively expressed.

Vascular cell adhesion molecule (VCAM-1) and endothelial leukocyte adhesion molecule (ELAM-1) are involved in modulating leukocyte-endothelial adhesion. They are normally not expressed on endothelial cells, but like ICAM-1 are induced by proinflammatory cytokines (Munro et al, 1989).

Two studies showed that monoclonal antibodies to ICAM-1 reduce infarct size and improve neurological outcome in reversible spinal cord ischaemia but not irreversible brain ischaemia

(Clark et al, 1991; Bowes et al, 1993). In a more clinically relevant study of transient MCA occlusion in a rat model, administration of an ICAM-1 antibody prior to reperfusion resulted in decreased infarct size, decreased polymorph infiltration and improved neurological outcome (Zhang et al, 1994).

A circulating soluble form of ICAM-1 (cICAM-1) has been characterized and levels of cICAM-1 shown to be significantly lowered in stroke patients (Clark et al, 1993) suggesting that these patients have altered leukocyte adhesion function.

Platelet-endothelial cell adhesion molecule-1

Platelet-endothelial cell adhesion molecule-1 (PECAM-1; CD31) is expressed in large quantities at the junctions between endothelial cells and on the surfaces of monocytes and polymorphs. Antibodies to PECAM-1 block leukocyte transmigration, suggesting that this molecule is required for neutrophil transmigration in vivo and thus may have a role in ischaemia/ reperfusion (Muller et al, 1993, Vaporciyan et al, 1993).

1.1.8 Platelet adhesion and aggregation

Platelet adhesion to vascular endothelium and aggregation can occur in response to a variety of mechanical and chemical stimuli. Physiological agonists, such as thrombin, collagen and ADP may be present at the site of endothelial injury or activation, and stimulate platelet adhesion.

At levels of shear stress above 60-80 dynes/cm^2, stable platelet aggregation can occur without the need for exogenous agonists (Ruggeri, 1993). Recent studies have established that fibrinogen is the adhesive protein mediating platelet aggregation in a low shear environment and von Willebrand Factor (vWF) in a high shear environment. High shear stress results in binding of vWF to GPI_b and this induces a transmembrane influx of calcium ions, resulting in platelet activation (Chow et al, 1992). Following platelet activation, the receptor $\alpha IIb\beta 3$ is able to bind with soluble adhesion proteins e.g. fibrinogen, and hence mediate platelet-platelet interactions (=aggregation).

Von Willebrand Factor is required for both platelet activation and platelet adhesion in shear-induced aggregation. Two distinct sites in the large vWF molecule appear to support this binding.

The initial adhesion of platelets *in-vivo* to a thrombogenic surface involves platelet interaction with exposed adhesive proteins in the vessel wall. This is quite different to *in-vitro* platelet-platelet interactions, due to the differing affinities of platelet receptors for ligands depending upon whether the particular ligand is immobilised or in solution (Ruggeri, 1993). Similarly, receptor specificity also depends on whether or not platelets are activated. For example, the $\alpha IIb\beta 3$ receptor in activated platelets can interact with fibrinogen, vWF, fibronectin and vitronectin (all containing the RGD sequence). However, on non-activated platelets, $\alpha II_b\beta 3$ can only interact with immobilised fibrinogen (Savage, 1991).

Platelet GPI_b can bind to surface vWF with or without shear stress. Thus the two platelet receptors GPI_b and $\alpha II_b\beta 3$ are involved in two separate pathways leading to the attachment of unstimulated platelets to thrombogenic vessel wall which present fibrinogen and/or vWF to flowing blood (Ruggeri, 1993).

Thrombus formation occurs following platelet-surface contact which leads to platelet "spreading", a conformation change of the platelet which makes adhesion irreversible. Spreading involves surface- bound RGD containing ligands with $\alpha II_b\beta 3$ (Pytela et al, 1986). Platelet activation then results in the binding of additional platelets to the thrombus.

It is possible that additional platelet receptors (such as GPI_a-II_a) and vascular ligands (e.g. fibrinogen, fibronectin and vitronectin) may also play a role.

Integrin $\alpha II_b\beta 3$ has been shown to serve as a receptor for 4 proteins: fibrinogen (Bennett et al, 1983), fibronectin (Gardner and Hynes, 1985), von Willebrand Factor (Ruggeri et al, 1982), and vitronectin (Pytela et al, 1986). All 4 proteins contain the RGD sequence, which is the key recognition domain involved in their binding to platelets. Thrombospondin and collagen may also bind to the $\alpha II_b\beta 3$ complex. The final common pathway of platelet activation by several physiological stimuli (eg. thrombin, collagen, ADP and shear stresses), is the binding of fibrinogen to $\alpha II_b\beta 3$ (Coller et al, 1983).

Following platelet activation and spreading commencing, the activated platelet becomes a target for other platelets, and a platelet plug is formed from these platelet-platelet interactions. Platelet adhesion in the venous circulation is not shear-dependent and thus GPIb and vWF are not involved. Both activated and unactivated platelets can be involved. Several platelet integrins, GPIa-IIa ($\alpha 2\beta 1$), $\alpha v\beta 3$, and $\alpha IIb\beta 3$ mediate platelet adhesion.

Platelet-rich thrombi have been shown to be resistant to lysis by thrombolytic agents and have thus become targets for adjunctive thrombolytic therapy (Falk, 1992). Arterial reocclusion after effective thrombolysis appears to be largely due to platelet interaction with the affected arterial surface which stimulates adhesion, aggregation and rethrombosis (Golino et al, 1988).

Platelet-activating factor (PAF)

Platelet-activating factor (PAF) is a high molecular weight phospholipid released from platelets, neutrophils and vascular endothelium. PAF is involved in leukocyte adhesion to vascular endothelial cells which have been stimulated with thrombin or histamine. Endothelial co-expression of P-selectin and PAF appear to maximise PMN adhesion (Lorant et al, 1991). PAF-receptor antagonists have been shown to decrease reperfusion injury in a myocardial model (Squadrito et al, 1993; Ioculano et al, 1994).

1.1.9 Current model of neutrophil adhesion to endothelial cells in ischaemia/ reperfusion

Margination and adhesion through specialised receptor molecules are a prerequisite for leukocyte accumulation in ischaemic and reperfused tissue. Leukocyte adhesion to post-capillary venules appears to be the major event occuring in the "no reflow" phenomenon during reperfusion of ischaemic tissues.

Chemotactic factors which may enter the bloodstream by diffusion through the vessel wall remain close to the endothelial cell, and thus marginated cells are more likely to become activated and adherent. This model is based on the reviews of Butcher (1991), and von Adrian and Arfors (1993).

Step 1. **Reversible "rolling" of leukocyte mediated by selectins.**

Within minutes of tissue injury, neutrophils "roll" along vessels in areas of tissue injury or ischaemia. "Rolling" almost certainly involves rapidly reversible receptor-ligand interactions of neutrophils to post-capillary venular walls and is mediated by selectins. This initial adhesion event involved selectin binding to oligosaccharide ligands such as sialyl Lewis^x (sLe^x). Neutrophil L-selectin is constitutively expressed at high levels on circulating non-activated neutrophils and mediates the binding of neutrophils to cytokine-activated endothelial cells (EC). "Rolling" slows the passage of neutrophils through capillaries and

post-capillary venules, allowing the neutrophil to be exposed to the endothelial cell surface where more permanent binding can occur. Rolling appears to be essential for successful neutrophil-EC interaction.

Systemic treatment with antibodies to L-selectin (but not with antibodies to β2 integrins) has been shown to decrease rolling in mesenteric venules by 70-80% in rabbits (von Adrian et al, 1991). Similarly, human neutrophils injected into rabbit superior mesenteric artery roll in inflamed post-capillary venules via an L-selectin dependent mechanism (von Adrian et al, 1992). The endothelial selectins, E- and P-selectin also appear to support rolling. These selectins are induced on ischaemic (or inflamed) endothelium and bind sLe^x and related surface carbohydrate ligands expressed on leukocytes in vitro (Lawrence and Springer, 1991; Bevilacqua et al, 1989).

Thus there appears to be a bi-directional recognition mechanism of leukocyte rolling. L-selectin can interact with endothelium independent of endothelial sLe^x by recognising an endothelial ligand (a carbohydrate) and simultaneously presenting sLe^x to vascular selectins.

Step 2. **Activation of rolling neutrophils in the vascular lumen.**

Neutrophils which have been activated by chemoattractants shed L-selectin from their cell surface (Kishimoto et al, 1989). The β-2 leukocyte integrin, Mac-1, is rapidly upregulated during neutrophil interactions with inflamed venular endothelium. The specific factors responsible for activation of rolling neutrophils are not well-characterised. The release of histamine and thrombin from mast cells, and cytokines (IL-1 and TNF-α) from endothelium, lead to release of chemotactic cytokines such as IL-8 and increased expression of E-selectin and ICAM-1.

Step 3. **Stable binding to the endothelium involves the activation-dependent β2 integrin, Mac-1 (CD11b, CD18).**

Leukocyte β2 integrins, in particular Mac-1, are believed to be unable to bind their endothelial ligand until they are activated. Cell activation appears to result in a temporary conformational change of the molecule. Following activation, exposure to an inflammatory stimulus results in firm leukocyte attachment to the endothelium via Mac-1 to ICAM-1 binding. Stable binding of leukocytes to endothelium can only take place when the leukocytes are "rolling". Neutrophils lacking L-selectin or sLe^x roll poorly and cannot stick in response to chemotactic stimulation (von Adrian et al, 1992b). However, Arfors et al

(1987) showed that anti-β2 antibodies allowed release of rolling neutrophils to the circulation, by preventing the arrest and stable attachment of rolling cells.

Activation-dependent adhesion via leukocyte integrins is stable under physiological shear forces and sustained for several minutes, but is eventually reversible.

This multi-step model of recognition permits a given receptor to be involved in more than one recognition event whilst still maintaining specificity. Both selectin-dependent early adhesion and activation-induced β2 integrin function are essential for firm neutrophil attachment to ischaemic endothelium (von Adrian and Arfors, 1993).

1.1.10 Activation of the blood complement system

Activation of the complement system by the classic or alternative pathways results in the production of the pro-inflammatory mediators C3a, C4a and C5a, which recruit leukocytes to sites of tissue injury (Fernandez et al, 1978). C5a has also been shown to induce cytokine synthesis and release from leukocytes and monocytes (Ember et al, 1994; Scholz et al, 1990; Okusawa et al, 1987; Okusawa et al, 1988).

1.1.11 Cytokines

Cytokines are polypeptides or glycoproteins of low molecular weight. Most cytokines are not stored as preformed molecules; hence their production requires new gene transcription and translation. Transcription is dependent upon an unstable mRNA, allowing for tight control of expression.

All cytokines exert their effect by binding to specific cell-surface receptors. Their ability may also be modulated by expression and shedding of cytokine cell-surface receptors. Vascular endothelial cells are both a target and a source of cytokines.

In a rat model of coronary ischaemia/ reperfusion, tumour necrosis factor (TNFα) was not detected during ischaemia of one hour duration, but was expressed following reperfusion. Antibodies to TNFα resulted in decreased CPK and myeloperoxidase activity (Squadrito et al, 1993).

Ischaemia/reperfusion of rat hind limb has been shown to result in local as well as systemic effects: increased muscle permeability and intramuscular haemorrhage, and increased lung permeability, haemorrhage and increased tissue myeloperoxidase activity reflecting white cell accumulation in the lung tissue. Within 60 minutes of reperfusion, increased levels of TNFα, IL-1 and IL-6 were noted. Antibodies to TNFα protected the lung and skeletal muscle from these injuries, confirming the role of cytokines in mediating local and remote effects of tissue injury (Seekamp et al, 1993).

Interleukin-1

Interleukin-1 (IL-1) has been shown to induce endothelial synthesis of procoagulant Tissue Factor, and increased adhesiveness of leukocytes to endothelial cells, which suggests a major role in reperfusion injury as well as inflammation (Bevilacqua et al, 1985).

Hypoxia induces IL-1 mRNA and release of IL-1 by cultured endothelial cells. IL-1 stimulates induction of ELAM-1 and ICAM-1 on endothelial cells, supporting the hypothesis that cytokine production by hypoxic endothelial cells can contribute to the host response in the pathogenesis of ischaemic vascular injury (Shreeniwas et al, 1992).

Brain IL-1β mRNA has been shown to be elevated acutely after permanent focal ischaemia (particularly in hypertensive rats), suggesting that this potent cytokine may have a role in brain damage following ischaemia. Expression is up-regulated within one hour of ischaemia and there is a major up-regulation from 6 hours to 5 days following onset of brain ischaemia (Liu et al, 1993). The degree of up-regulation was related to the severity of neurological deficit, but the cellular origin of this cytokine was not clarified in this study (in situ hybridisation would be required to obtain cellular localisation of expression). Transient focal cerebral ischaemia has also been shown to up-regulate IL-1 mRNA (Minami et al, 1991). The probable consequences of up-regulation of IL-1β mRNA and subsequent production of IL-1β include activation of microglia and astrocytes and transformation of endothelium to a prothrombotic state that predisposes to thrombosis.

Interleukin-8

Interleukin-8 (IL-8) is secreted by activated macrophages and granulocytes and by endothelial cells. Both forms stimulate pro-inflammatory responses in neutrophils, including superoxide radical production, adhesion and chemotaxis via binding to cell surface receptor coupled to intracellular signal transduction pathways (Holmes et al, 1991). However, IL-8 decreases

attachments of granulocytes to stimulated human umbilical vein endothelial cells (HUVECs) (Gimbrone et al, 1989). IL-8 given intravenously reduces granulocyte recruitment to inflammatory sites induced in rabbit skin (Hechtman et al, 1991) and anti-inflammatory effects have been observed in a model of myocardial I/R in the rabbit (Lefer et al, 1991) (= LAI^3 effect).

IL-8 (like other chemoattractants) induces rapid shedding of L-selectin from human granulocytes and thus may impair leukocyte rolling due to loss of L-selectin. This may be responsible for decreased granulocyte recruitment in vivo (Ley et al, 1993).

1.1.12 Heat Shock Proteins

Heat shock proteins play an important role in protecting cells against ischaemic injury. In a rat model of cerebral ischaemia, fixed ischaemia resulted in moderately increased Heat Shock Protein 70 (hsp70) and cfos mRNA expression. However reperfusion for 2 hours following 1 hour of ischaemia resulted in intense hsp70 mRNA expression, suggesting reperfusion triggered expression in previously ischaemic regions (Welsh et al, 1992). Similarly, An et al. (1993) demonstrated induction of cfos and cjun family mRNA immediately after reperfusion of focal cerebral ischaemia, peaking at 60 minutes.

Other studies have shown hsp70 mRNA and hsp70 are detected in endothelial cells, even in the ischaemic infarct zone, suggesting that inflammatory cells of non-neuronal origin may account for cytokine mRNA expression (Kinouchi et al, 1993). Thus immunohistochemical staining, Northern blot RNA assay and in situ hybridisation are needed to fully clarify the cellular origin of up-regulated cytokine and stress protein expression in I/R.

1.1.13 Endothelial modulation of the coagulation system in ischaemia/reperfusion. Anticoagulant function of endothelium

The vascular endothelium is an actively anticoagulant and antithrombotic surface. A glycocalyx "carpet" lies on the luminal surface of endothelium, regulating access to surface receptors. The glycocalyx is extremely sensitive to hypoxia and its disruption may be the first sign of endothelial cell damage. There appears to be a relationship between preservation

of the glycocalyx and endothelial cell integrity (hence capillary permeability) (Ward and Donnelly, 1993).

The thrombomodulin - protein C - protein S system contributes to the anticoagulant function of endothelium. Thrombomodulin binds and alters thrombin resulting in fibrinogen no longer being its substrate, but instead the thrombomodulin-thrombin complex activates protein C which inactivates factors V and VII as well as tissue- type plasminogen activator inhibitor.

Tissue-type plasminogen activator (tPA) is also released from the endothelium.

Tissue-type plasminogen activator

Tissue-type plasminogen activator is a serine protease which catalyses the conversion of plasminogen to active plasmin by hydrolysis of the peptide bond between Arg 560 and Val 561 in the plasminogen molecule. Tissue- type plasminogen activator is produced as a 527 amino acid polypeptide, which displays homology with parts of other proteins. It is built from 5 domains. From the N-terminus end, it consists of a finger domain (homologous to a similar fibronectin domain), a growth factor domain, two kringle domains (originally found in prothrombin) and a protease domain (Pennica et al, 1983).

Regulation of tPA synthesis appears possible via protein kinase C, cAMP and steroids, and may be associated with the regulation of type-1 plasminogen activator-inhibitor (PAI-1) synthesis.

Endothelium releases tPA continuously and acutely in response to stimulating factors. Vascular endothelial tPA activity has been demonstrated in smaller veins and venules and in the vasovasorum of larger veins and arteries, and in the pulmonary vessels (van Hinsbergh, 1988).

Utilizing in-situ hybridisation techniques on a panel of 14 tissues from primates, tPA mRNA was demonstrated in the endothelial cells of small venules and in the adventitia of larger vessels (Gordon et al, 1989). Acute release of tPA occurs in response to a variety of stimuli such as products from platelet and coagulation activation (Emeiss, 1988). There appears to be a significant intracellular and extracellular pool (on binding sites on the endothelium) of tPA.

Tissue-type plasminogen activator release associated with coagulation sytem activation occurs both locally and downstream of the site of fibrin formation.

Fibrinolysis depends not only on the supply of tPA in the circulation but also on the local endothelial tPA activity in response to stimuli such as reaction products from coagulation e.g. activated Xa (Giles et al, 1989). Local release provides high concentrations of tPA at the site of fibrin formation. Only tPA incorporated during fibrin formation is an efficient fibrinolysis activator, having relatively low activity in pre-existing fibrin clots.

Procoagulant functions of endothelium

In response to a variety of stimuli, including mechanical trauma, ischaemia and inflammation, endothelium can become procoagulant.

The procoagulant behaviour of endothelium includes:

(1) expression of plasminogen activator inhibitor-1 (PAI-1)

(2) expression of binding sites for factors IX and X and platelet adhesion molecules. Factor X has been shown to be induced in endothelial cell culture exposed to hypoxia, supporting a role of hypoxia in inducing endothelial procoagulant activity (Ogawa et al, 1991).

(3) down regulation of anticoagulant mechanisms

(4) expression of tissue factor (TF) which activates the extrinsic pathway of coagulation on exposure to factor VII/VIIa.

Plasminogen activator inhibitors

Type I plasminogen activator inhibitor (PAI-1) is the physiological inhibitor of both tissue-type and urinary-type plasminogen activators. It is a single chain glycoprotein consisting of 379 amino acids (Erickson et al, 1984). Unbound PAI-1 has a short half-life (7 minutes in rabbits [Colucci et al, 1985]), however binding to vitronectin stabilises PAI-1 in its active conformation and thus increases its half-life (Loskutoff et al, 1989).

PAI-1 is derived from endothelial cells, hepatocytes and alpha granules of platelets (Erickson et al, 1984), and its activity is increased by interleukin-1 (Nachman et al, 1986), TNF, TGFβ and LPS (Sawdey and Loskutoff, 1991). Some disease states have demonstrated increased PAI-1 expression, e.g. renal disorders, which may be mediated by endotoxin (Keeton et al, 1993). Cytokine release in ischaemia/ reperfusion may result in elevated PAI-1 levels and thus contribute to increased endothelial procoagulant activity and increased thrombin formation in vessels.

Activation of the coagulation system

Formation of thrombin is central to the coagulation cascade. The most important source of thrombin quantitatively is platelets. Endothelium is also capable of generating thrombin which could prime the platelets to release their stores. However, on "resting" endothelium and in the absence of TF expression, endothelial thrombin is inactivated by antithrombin III or bound by thrombomodulin. If cytokines or other mediators cause down-regulation of thrombomodulin and up-regulate expression of TF, coagulation occurs rapidly.

Tissue factor

Tissue Factor (TF) is a 47 kDalton membrane-bound glycoprotein.

It is the cofactor for factor VII and VIIa, which in its absence have no activity. Binding is calcium-dependent. The TF-VIIa complex causes the activation of fators IX and X. Rapid preferential activation of factor VII bound to TF by trace amounts of Xa is a key early step in the extrinsic coagulation cascade (Rao and Rappaport, 1988).

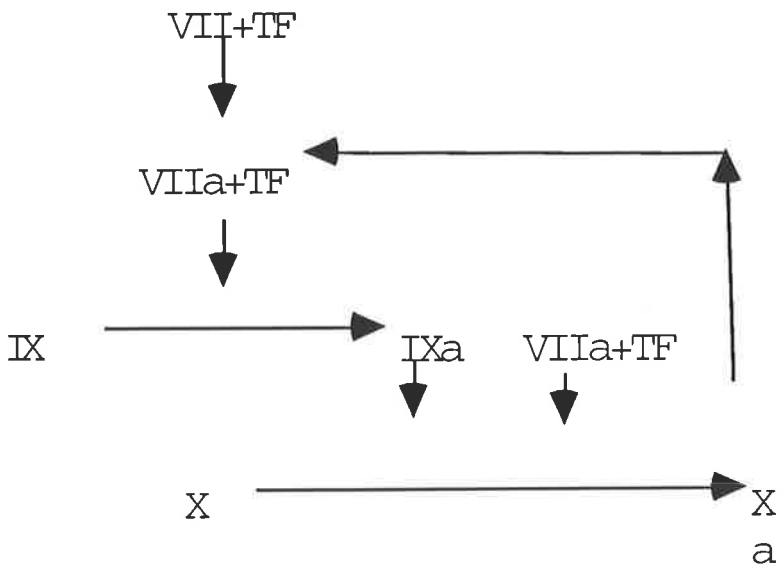


Figure 1.1 Activation of the extrinsic pathway:

(based on Rao and Rappaport, 1988)

Tissue Factor is present in the plasma membrane of many cells and is found in the adventitia and to a lesser extent, the media of normal blood vessels (Wilcox et al, 1989; Drake et al, 1989).

In cerebral tissue of primates, TF was found most prominently in cerebral cortex, and in progressively lower amounts in basal ganglia, cerebellum and white matter. TF was also

demonstrated to be associated with non-capillary microvessels in the basal ganglia and cortex (del Zoppo et al, 1992). A study in this laboratory using a monoclonal antibody to TF (TF9-6B4) in focal cerebral ischaemia and reperfusion showed a significant increase in microvascular patency in the 7.5-30 μm and 30–50 μm diameter vessels consistent with blocking exposed TF in reperfused post-capillary venules and larger venules (Thomas et al, 1993). In a rabbit large vessel injury model, TF antibody decreased the incidence of platelet-mediated arterial thrombosis (Jang et al, 1992).

These findings suggest that microvascular thrombosis may occur as a result of exposure of blood to subendothelial tissue factor in situations of vascular injury or events resulting in increased endothelial permeability (such as ischaemia/reperfusion).

Using cultured vascular endothelium, a number of cytokines, including TNF (Nawroth and Stern, 1986; Bevilacqua et al, 1986), IL-1 (Nawroth et al, 1986; Bevilacqua et al, 1986) and bacterial lipopolysaccharides (LPS) have been shown to induce TF activity (Colucci et al, 1983; Moore et al, 1986). Bevilacqua et al (1986) showed that the effects of TNF and IL-1 were additive in human vascular endothelium. The peak procoagulant response was noted 4 hours after cytokine stimulation.

The mechanism of TF activation appears to be due to increased mRNA levels which were detectable within 30 minutes of exposure to LPS and maximal at 2 hours. However, this observation was felt to be largely due to increased TF mRNA stability rather than an increased rate of transcription (Crossman et al, 1990). Maximal levels in response to TNF have been noted at 4 hours (Conway et al, 1989).

Inflammatory stimuli (e.g. LPS) have also been shown to induce TF expression in macrophage/monocytes via gene transcription within 15 minutes, peaking at 2-4 hours (Mackman et al, 1993). Thus expression of TF on the surface of monocyte/macrophage cells and exposure of subendothelial tissue would lead to formation of the TF-factor VIIa complex and hence thrombin generation and fibrin formation.

Tissue factor mRNA expression in cerebral tissue has been noted in astrocytes in the cortex and Bergmann glia (cerebellum). Endothelial cells did not express TF under resting conditions or following stimulation with LPS, suggesting that endothelial cells in vivo respond differently to cytokine stimulation than cultured endothelial cells (Mackman et al, 1993). The role of astrocytes in the formation of the tight junctions between endothelial cells

that form the blood-brain barrier suggests that TF may form a "haemostatic envelope" around vessels in the brain, which can activate coagulation when vascular integrity is disrupted (Drake et al, 1989).

No data are available using in-vivo models to clarify whether TF is up-regulated during I/R in brain, or whether medial and adventitial TF is exposed to circulating blood elements during I/R, thus providing the basis for the in situ hybridisation experiments undertaken.

Thrombomodulin

Cytokines and LPS also reduce thrombomodulin (TM) activity (Moore et al, 1986; Nawroth et al, 1986) by suppressing transcription (Conway and Rosenberg, 1989). Thrombomodulin is a membrane-bound co-factor which causes the activation of Protein C (APC) in the presence of thrombin. APC with its co-factor, Protein S, has an inhibitory effect on coagulation by cleaving VIIa and Va. Thrombomodulin has not been demonstrated in cerebral microvasculature. If demonstrated in cerebral microvasculature, these responses would facilitate the rapid conversion of microvascular endothelium from anticoagulant to procoagulant status following cytokine or hypoxic stimulation during ischaemia/ reperfusion.

Chapter Two

Study of microvascular patency following 4 hours of focal cerebral ischaemia and no reperfusion in comparison to 3 hours of ischaemia and one hour of reperfusion.

2.1 Aims

A considerable amount of microvascular patency data are available for focal ischaemia followed by periods of reperfusion. However, no patency data has been obtained in focal cerebral ischaemia and no reperfusion.

Obtaining a data set of microvascular patency following 4 hours of ischaemia and no reperfusion would be useful in several ways:

(1) To compare the effects of 1 hour of reperfusion compared to an extra hour of ischaemia on microvascular patency.

(2) As a baseline for further studies involving pharmacological agents which may modify intravascular thrombosis thus affecting patency and potentially modifying infarct size.

2.2 Null Hypothesis

The null hypothesis of this study states that there is no difference in relative microvascular patency in ischaemic basal ganglia between animals undergoing 4 hours middle cerebral artery (MCA) occlusion and those undergoing 3 hours middle cerebral artery occlusion with 1 hour reperfusion.

2.3 Materials and methods

2.3.1 Focal cerebral ischaemia /reperfusion model

The focal cerebral ischaemia/reperfusion model described below has been used extensively in this laboratory for two major types of studies: (1) immunohistochemistry, and

(2) microvascular patency studies.

Adolescent male baboons (Papio anubis/cyanocephalus) weighing between 7.5 and 10.0 kg were used in these studies. These animals were observed to be free of disease during mandated United States Federal and State Government quarantine periods (60 days in total). All procedures were approved by the Animal Research Committee of Scripp's Research Institute and were performed in strict adherence to the standards published by the National Research Council (the Guide for the Care and Use of Laboratory Animals), the National Institutes of Health policy on Humane Care and Use of Laboratory Animals and the USDA Animal Welfare Act.

The techniques currently used in this model are closely based on those previously reported from this laboratory (del Zoppo et al, 1991). General anaesthesia was induced in the subjects by an initial sedating dose of ketamine, followed by intravenous pentothal sodium (10 mg/kg bolus and 10 mg/kg/hr) and halothane. Endotracheal intubation was then performed. Under antibiotic cover, a right transorbital approach was made to the internal carotid and middle cerebral arteries. This procedure involved removal of the medial sphenoid wing. A small inflatable silastic balloon assembly (Mentor Corporation, Goleta, California) was placed around the right middle cerebral artery (MCA) proximal to the origin of the lenticulostriate arteries. The device was fixed in position using vicryl ties in such a way that the MCA sat between the inflatable balloon and a coated wire frame. The device was fixed in position using methylmethacrylate and the connecting tube and plug were placed subcutaneously in a tunnel near the apex of the head.

Postoperative recovery was closely monitored and typically the subject was fully conscious within 2-4 hours. The animals were closely observed and assessed neurologically to ensure that there was no deficit related to the procedure. Observation was continued for a period of 6-7 days prior to any further procedure being performed. A normal neurological score (N=100), using the scale devised by Spetzler et al (1980) (see Appendix I) was observed prior to proceeding to middle cerebral artery occlusion.

In focal ischaemia procedures, occlusion of the right middle cerebral artery was performed by inflation of the occlusion catheter. The awake baboon was seated in a chair and the subcutaneously placed plug and tube were exposed under local anaesthesia (1% lignocaine). The appropriate volume of fluid (approximately 150 µls) was then injected into the device. The tubing was then clamped while the plug was reinserted into the device. No distress or

agitation was noted in any animal as the neurological deficit became apparent. The skin incision was then closed. Neurological scores were performed at 10 minutes, 1 hour and each subsequent hour. In all subjects, a definite neurological deficit was noted within 10 minutes. Full blood counts were obtained at 0, 60, 180 minutes of ischaemia and at intubation prior to perfusion. In procedures in which reperfusion was performed, the fluid injected into the device was aspirated at the appropriate time.

Approximately 15 minutes prior to the end of the period of reperfusion, each animal underwent perfusion-fixation under general anaesthesia. Intubation was peformed under ketamine which was followed by pentothal (10 mg/kg). The subject was then mechanically ventilated.

In animals not undergoing a period of reperfusion, the MCA occlusion device was deflated just prior to perfusion of the brain. A median sternotomy was performed and the descending aorta and inferior vena cava exposed. These vessels were both clamped and the right atrial appendage was vented. Cerebral perfusion-fixation was commenced by left ventricular cannulation and infusion of a chilled solution (4°C) of plasmalyte at a pressure of 160-200 mm Hg. The solution contained 25 grams/litre of bovine serum albumen adjusted to 340 mOsm with NaCl to which was added 2500 units of heparin / litre and 7 μM Na^+ nitroprusside. Four litres of perfusion fluid were infused over a 5 minute period.

(a) Vessel Patency Studies

Subjects used for vessel patency studies (i.e. 4 hour ischaemia and TP9201 study) were perfused via the left ventricular cannula with plasmalyte solution followed by chilled carbon suspension in paraformaldehyde (India ink, Pelican AG, Hannover, Germany in 4% paraformaldehyde). Perfusion pressure was maintained at 180-200 mmHg for this procedure due to greater fluid viscosity. Seven litres were perfused over a 15 minute period. This technique resulted in brain fixation by the paraformaldehyde and carbon tracer filling of all patent vessels.

The brain was then removed and underwent a period of 7 days fixation in AFA solution (87% ethanol, 10% of 4% formaldehyde and 3% glacial acetic acid), prior to coronal sectioning at 5 mm intervals.

Visual inspection of the brain was undertaken for macroscopic evidence of decreased perfusion and evidence of haemorrhage. Photographs of coronal sections were taken and

diagrammatic representations made. Blocks were then cut of identical sites of both basal ganglia and temporal cortex. The left side (not ischaemic) served as a control. Twenty consecutive sections were cut at 10 μm thickness by Hector Herreira (Department of Cytology, Sharp Hospital, San Diego, California) and stained with basic fuschin/methylene blue.

(b) Immunohistochemical studies

Subjects used for immunohistochemical studies underwent brain perfusion with chilled plasmalyte solution, as described previously, and then rapid removal of the entire brain and upper spinal cord from the cranium. The brain was then kept on ice and sliced coronally in 5-6 mm thick sections. Blocks of right and left basal ganglia, temporal cortex and parietal cortex were cut - each block ≈ 1 x 1 x 0.5 cm. The blocks were placed in a cassette, covered with OCT and snap frozen in pentothal containing dry ice. These sections were then stored at -70°C for sectioning and subsequent immunohistochemical staining. These immunohystochemical experiments were the projects of Y. Okada M.D. and G. Hamann M.D. and are briefly described in Appendix 4. The staining and counting of sections was undertaken by these colleagues.

(c) Measurement of Microvascular Patency

The sections of brain perfused with india ink were viewed to exclude artefact and adequacy of staining and vessel filling. Every third section (30 μm apart) of both left and right basal ganglia of each animal was then counted . Thus 7 sections were counted from both basal ganglia of each animal.

Vascular patency was quantified using the VIDAS video imaging system. A Hamamatsu C2400 video camera (Hamamatsu, Japan) was connected to a light microscope (Carl Zeiss, Munich, Germany) with a powered stage. The imaging system measured the number and minimum diameter of all vessels containing India ink in the fields studied. Sections were viewed to ensure good perfusion and absence of artefacts. The area of study was selected to contain the maximum proportion of grey matter, as the white matter contains several-fold fewer vessels than adjacent grey matter. The system measured 90 fields (9x10) at 200 times magnification in a 5x5 mm area of each slide.

A minimum of 2000 vessels per left basal ganglia were measured, although generally 3000-5000 vessels were counted in the 7 slides, and a size and frequency profile obtained.

The data were converted to a MacIntosh compatible format. Using the "Wingz" programme on a Quadra 700 computer, the data derived from each section were sorted into ascending diameter measurements. Values less then 4 μm were deleted and the remaining values put into bin ranges of 4-7.5 μm (representing capillaries), 7.5-30 μm (representing precapillary arterioles and post-capillary venules), 30-50 μm (larger arterioles and venules) and 50-100 μm (arterioles and venules - few vessels were measured in this category).

Using the data obtained for the sections of left and right basal ganglia, a percentage reflow was able to be calculated at each vessel size range for each animal.

The reflow for each size range was calculated as the total number of vessels counted in 7 sections in the particular size range on the right (ischaemic) side divided by the total number of vessels counted in 7 sections in the particular size range on the left (control) side, expressed as a percentage.

The actual number of vessels in the control side varied quite significantly between subjects, and in particular several brains contained vessels which appeared to be helical in orientation, resulting in a greater number of vessels being counted. This appearance was also noted upon examination of cortical blocks in the same animals.

2.4 Reproducibility of video imaging system

Reliability and reproducibility data have been previously reported with this video-imaging system (del Zoppo et al, 1991). Reproducibility of the measurements was assessed by recounting eight separate microvessels 4-24 μm in minimum diameter on twenty occasions. These vessels were independently measured on a 10 μm reticule. Coefficients of variance were 0, 0.18, 0.47, 0.54, 0.89, 5.46, 5.74, and 5.93% and were independent of vessel diameter.

Reliability of microvascular patency was determined by counting 90 fields in 20 successive sections (as previously described) of left basal ganglia of 2 specimens. The distribution of vessel diameters from 4 to 100 μm showed no significant difference between sections up to 50 μm apart.

Sections were counted from the same subject alternately from left and right basal ganglia by one individual. The contrast and intensity of the video-imaging system was carefully

adjusted to ensure that only carbon-filled vessels were counted and that pyknotic nuclei were excluded.

2.5 Results

The four animals undergoing 4 hour middle cerebral artery occlusion and no reperfusion (MCAO) were #176, 202, 207, and 175. The animals which had previously undergone 3 hour middle cerebral artery occlusion and 1 hour of reperfusion (MCAO/R) were #107, 130, 142, and 144.

2.5.1 Clinical findings

Clinical and haematological data are shown in Tables 2.1 and 2.2.

Subjects in both groups underwent MCA occlusion device placement 7 days prior to the experiment and recovered with no neurological deficits (N=100). Following MCA occlusion, all subjects sustained a persisting neurological deficit (N=46, 42, 27, 76; mean 48) consistent with a focal cerebral ischaemic event. The previously studied MCAO/R subjects sustained similar persisting neurological deficits (N=47, 55, 81, 81; mean 66).

Baseline white cell count was 8 500, 8 800, 9 800, and 10 000 per μL (mean 9 300) in the MCAO group, which became elevated during the ischaemic event. Similarly, the MCAO/R group had baseline white cell counts of 11 600, 13 000, 13 300 and 14 100 (mean 13 000) which also became markedly elevated during the ischaemic period.

2.5.2 Macroscopic findings

Photographs of coronal sections of brain are shown in Appendix 2.

Gross examination of the MCAO/R subject brains demonstrated one specimen with a small petechial haemorrhage only. All subjects had evidence of right corpus striatal perfusion defects manifest by pallor in the right basal ganglia. In the MCAO group a single animal (#175) had a large haemorrhage involving the entire right basal ganglia. This is evident from the photograph. Because of the presence of the extensive haemorrhage, it was not possible to

quantitate microvascular patency, so that this specimen was removed from the data acquisition. Two other subjects had petechial haemorrhage, but all three of the remaining subjects had evidence of pallor to carbon tracer in the right basal ganglia.

2.5.3 Microvascular findings

Tables 2.3 to 2.6 detail the number of patent microvessels (manifested by those containing carbon tracer) for the given microvascular sizes on each counted slide. The microvessels of 4.0 to 7.5 μm diameter represent capillaries, 7.5 to 30.0 μm postcapillary venules and precapillary arterioles, those 30.0 to 50.0 μm small arterioles and venules, and 50.0 to 100.0 μm larger arterioles and venules. The microvascular size groups represent cumulative counts from each of seven slides 30 μm apart in each of the left and right basal ganglia for all specimens. The total numbers of vessels counted for each slide are noted in the last column of each table.

3 hour ischaemia / 1 hour reperfusion cohort

Tables 2.5 and 2.6 document the number of vessels counted in each slide of this group of subjects.

Percentage reflow for each size range was calculated as number of patent microvessels in ischaemic basal ganglia divided by number of patent microvessels in control basal ganglia for the same microvascular size.

A mean 45% reflow was observed in microvessels 4.0 to 30.0 μm diameter, with higher reflows evident in larger vessels. These were due to the smaller number of 50.0 to 100.0 μm vessels for counting in each section. Those observations were consistent with previous observations in other cohorts. A more detailed examination of the data demonstrates that two subjects (#142, #144) had a 28 and 47% reflow in the 4.0 to 7.5 μm range, but a >60% reflow in 7.5 to 30.0 μm range. Two remaining subjects (#130, #107) had a 44% and 64% reflow in the 4.0 to 7.5 μm range with only 17 to 29% in the 7.5 to 30.0 μm range. This suggests a clustering around 7.5 μm in those animals. The overall mean reflow was found to be 45.8 ± 12.6% in the 4.0 to 7.5 μm range and 44.6 ± 22.1% in the 7.5 to 30.0 μm vessel group (see Table 2.7).

Figure 2.1 shows relative microvessel patency for each animal and Figure 2.3 shows overall patency.

4 hour ischaemia / no reperfusion cohort

Vessel patency is given on Tables 2.3 and 2.4 and summary data in Table 2.7. Microvascular patency was assessed in 3 subjects (one excluded due to haemorrhage involving all of right basal ganglia).

In the 4.0 to 7.5 μm (capillary) range, mean patency was 54.7% (standard deviation 13.1%) a mean of 6,000 vessels were counted on the control side in this size range. In the 7.5 to 30.0 μm range (post-capillary venules and pre-capillary arterioles), mean patency was 69.1% (standard deviation 26.6%), with a mean of 5,900 vessels counted on the control side. In the larger vessel categories, % reflow was 94% (30-50 μm), 115% (50-100μm) and 187% (>100 μm), but the number of vessels in these categories is too small for statistical analysis. At each vessel size category, no statistical difference in microvascular patency was noted between the 3 hour ischaemia/1 hour reperfusion and the 4 hour ischaemia subjects (Figure 2.2).

2.6 Discussion

A considerable body of information regarding the "no-reflow" phenomenon has been undertaken in the laboratory in which this research fellowship was undertaken. A computerised video-imaging system has been utilised to calculate microvascular patency accurately. These patency studies have been undertaken exclusively using a 3 hour ischaemia followed by 1 hour of reperfusion paradigm.

The results of this study demonstrated that focal cerebral ischaemia of 3 hours duration followed by reperfusion, results in a relative microvessel patency in the ischaemic basal ganglia compared to the left (normal) side of 46% for the 4 - 7.5 μm diameter vessels, 45% for 7.5 - 30 μm vessels and 76% for the 30 - 50 μm diameter vessels. This was consistent with previous results. The relative microvascular patency following 4 hours of ischaemia were 55% for the 4.0 - 7.5 μm diameter vessels, 69% for the 7.5 - 30 μm vessels and 94% for 30 - 50 μm diameter vessels. The minimum number of vessels counted in the control basal ganglia of one animal was 3,973 vessels.

There was no significant difference in patency between these 2 study groups at any vessel size range.

Previous experiments using this 3 hour ischaemia / 1 hour reperfusion model have demonstrated a significant improvement in reflow in the 7.5 to 50 μm vessel size range, when the ischaemic brain is reperfused with blood containing Tissue Factor antibody (Thomas et al, 1993).

Similarly, using anti CD18 antibodies (blocking leukocyte adherence to endothelium), a significant increase in microvascular patency was found in the 7.5 to 50 μm vessel range (Mori et al, 1992). Neither study demonstrated a significant improvement in capillary patency and both studies found a mean 80% patency in the 7.5 to 30 μm vessel range.

These previous studies of Thomas et al, and Mori et al, demonstrated that reperfusion with modified blood (i.e. antiTF antibody, antiCD18 integrin antibody) following ischaemia/ reperfusion of 3/1 hours has a beneficial effect on the "no-reflow" phenomenon compared to reperfusion with unmodified blood in this model. However, the results of the present study suggests that 3 hours of ischaemia followed by 1 hour of reperfusion with "unmodified" blood and 4 hours of ischaemia are likely to be periods of ischaemic injury resulting in irreversible microvascular occlusions. The experiments conducted in chapter 3 involve the use of "modified" blood.

These data also provide a baseline for future studies with agents which may ameliorate microvascular occlusion without a period of reperfusion. However, one major deficit of this (and most animal models of reperfusion injury) is the inability to accurately assess the degree of neuronal injury and infarct size.

The possible effect of ischaemia/ reperfusion on vessel size has in part been addressed by the addition of sodium nitroprusside to the perfusate at the conclusion of each experiment. In each vessel group range from 4 to 50 μm, the percentage reflow remains relatively constant, suggesting marked vasodilatation or vasoconstriction does not occur. Garcia et al, suggested in 1983 that capillary expansion and rupture occur in zones of brain ischaemia and reperfusion, however this study did not utilise a sophisticated image analysis system and has not been replicated.

Table 2.1

4 Hour Ischaemia/ No Reperfusion

	175	176	202	207
Date of procedure	6/10/93	23/9/93	14/9/93	22/9/93
Neurological score				
Baseline	100	100	100	100
1 Hour	46	42	27	76
2 Hours	46	42	27	76
3 Hours	46	42	27	76
4 Hours	46	42	27	76
Full blood count				
Baseline				
Haemoglobin (gm/dL)	12.4	12.2	14.1	11.9
Platelets (per μL)	498,000	528,000	360,000	520,000
WBC (per μL)	8,800	10,000	8,500	9,800
1 Hour WBC	12,700	18,500	13,200	13,300
3 Hour WBC	25,000	34,500	-	17,800
Macroscopic findings:	large haemorrhage RBG	infarct RBG very small haemorrhage RBG	infarct RBG	infarct with small haemorrhage adjacent to block

Table 2.2

3 Hour Ischaemia/1 Hour Reperfusion

	107	130	142	144
Date of procedure	21/2/90	3/10/90	3/10/90	26/6/90
Neurological score				
Baseline	100	100	100	100
1 Hour	81	81	55	47
2 Hours	81	81	55	47
3 Hours	81	81	55	47
Reperfusion	81	81	55	47
Full blood count				
Baseline				
Haemoglobin (gm/dL)	14.1	13.0	13.3	11.6
Platelets (per μL)	502,000	479,000	483,000	509,000
WBC (per μL)	12,900	13,400	9,400	8,400
1Hour WBC	24,800	27,600	17,900	19,800
3 Hour WBC	25,500	25,700	17,700	32,700

Table 2.3

CONTROL (NON-ISCHAEMIC) LEFT BASAL GANGLIA - 3 HOURS ISCHAEMIA / 1 HOUR REPERFUSION						
SLIDE NUMBER	NUMBER OF VESSELS PER VESSEL SIZE (um)					
	4.0-7.5	7.5 - 30	30-50	50-100	>100	Total
Animal #130						
NMBG 3002	325.00	363.00	23.00	4.00	0.00	715.00
NMBG 3005	267.00	375.00	18.00	8.00	1.00	669.00
NMBG 3008	315.00	351.00	20.00	6.00	2.00	694.00
NMBG 3011	269.00	367.00	16.00	6.00	0.00	658.00
NMBG 3014	450.00	500.00	35.00	9.00	0.00	994.00
NMBG 3017	265.00	339.00	18.00	6.00	0.00	628.00
NMBG 3020	410.00	450.00	20.00	8.00	0.00	888.00
Total	2301.00	2745.00	150.00	47.00	3.00	5246.00
Animal #142						
NMBG 4205	216.00	265.00	17.00	1.00	0.00	499.00
NMBG 4208	190.00	203.00	14.00	6.00	0.00	413.00
NMBG 4211	224.00	255.00	13.00	4.00	0.00	496.00
NMBG 4214	390.00	304.00	30.00	3.00	3.00	730.00
NMBG 4217	413.00	323.00	22.00	5.00	2.00	765.00
NMBG 4219	389.00	303.00	22.00	2.00	2.00	718.00
NMBG 4220	282.00	215.00	8.00	1.00	0.00	506.00
Total	2104.00	1868.00	126.00	22.00	7.00	4127.00
Animal #144						
NMBG 4401	862.00	621.00	30.00	4.00	0.00	1517.00
NMBG 4404	833.00	630.00	24.00	1.00	0.00	1488.00
NMBG 4407	781.00	604.00	23.00	7.00	1.00	1416.00
NMBG 4410	797.00	643.00	33.00	4.00	0.00	1477.00
NMBG 4413	889.00	593.00	20.00	4.00	1.00	1507.00
NMBG 4417	944.00	592.00	25.00	4.00	0.00	1565.00
NMBG 4420	835.00	497.00	19.00	5.00	0.00	1356.00
Total	5941.00	4180.00	174.00	29.00	2.00	10326.00
Animal #107						
NMBG 8902	352.00	184.00	9.00	5.00	1.00	551.00
NMBG 8906	344.00	180.00	6.00	4.00	1.00	535.00
NMBG 8909	350.00	187.00	12.00	4.00	0.00	553.00
NMBG 8913	370.00	192.00	7.00	6.00	0.00	575.00
NMBG 8916	343.00	205.00	14.00	4.00	1.00	567.00
NMBG 8918	369.00	194.00	15.00	3.00	0.00	581.00
NMBG 8920	378.00	209.00	20.00	3.00	1.00	611.00
Total	2506.00	1351.00	83.00	29.00	4.00	3973.00

Table 2.4

ISCHAEMIC RIGHT BASAL GANGLIA - 3 HOURS ISCHAEMIA / 1 HOUR REPERFUSION						
	NUMBER OF VESSELS PER VESSEL SIZE (um)					
SLIDE NUMBER	4.0-7.5	7.5-30	30-50	50-100	>100	Total
Animal #130						
ISBG 3002	322.00	148.00	8.00	2.00	1.00	481.00
ISBG 3005	146.00	110.00	7.00	1.00	0.00	264.00
ISBG 3008	152.00	116.00	6.00	1.00	0.00	275.00
ISBG 3011	189.00	127.00	7.00	1.00	0.00	324.00
ISBG 3014	284.00	100.00	3.00	1.00	0.00	388.00
ISBG 3017	180.00	96.00	7.00	0.00	0.00	283.00
ISBG 3020	193.00	109.00	9.00	1.00	0.00	312.00
Total	1466.00	806.00	47.00	7.00	1.00	2327.00
Animal #142						
ISBG 4203	240.00	223.00	18.00	8.00	1.00	490.00
ISBG 4206	156.00	243.00	32.00	16.00	1.00	448.00
ISBG 4208	166.00	193.00	12.00	5.00	1.00	377.00
ISBG 4211	132.00	175.00	12.00	3.00	0.00	322.00
ISBG 4214	128.00	144.00	18.00	3.00	0.00	293.00
ISBG 4217	56.00	90.00	6.00	4.00	0.00	156.00
ISBG 4219	108.00	139.00	15.00	4.00	1.00	267.00
Total	986.00	1207.00	113.00	43.00	4.00	2353.00
Animal #144						
ISBG 4401	185.00	395.00	40.00	24.00	8.00	652.00
ISBG 4404	270.00	399.00	38.00	20.00	2.00	729.00
ISBG 4407	257.00	400.00	33.00	16.00	5.00	711.00
ISBG 4410	251.00	449.00	34.00	22.00	4.00	760.00
ISBG 4413	274.00	407.00	31.00	22.00	4.00	738.00
ISBG 4416	213.00	396.00	40.00	22.00	8.00	679.00
ISBG 4420	224.00	385.00	35.00	18.00	3.00	665.00
Total	1674.00	2831.00	251.00	144.00	34.00	4934.00
Animal #107						
ISBG 8902	92.00	27.00	3.00	3.00	0.00	125.00
ISBG 8905	171.00	28.00	5.00	2.00	0.00	206.00
ISBG 8908	165.00	34.00	5.00	3.00	1.00	208.00
ISBG 8912	167.00	33.00	4.00	3.00	1.00	208.00
ISBG 8915	189.00	32.00	5.00	2.00	1.00	229.00
ISBG 8918	126.00	35.00	5.00	4.00	1.00	171.00
ISBG 8920	204.00	36.00	5.00	3.00	1.00	249.00
Total	1114.00	225.00	32.00	20.00	5.00	1396.00

Table 2.5

CONTROL (NON-ISCHAEMIC) LEFT BASAL GANGLIA - 4 HOURS ISCHAEMIA / NO REPERFUSION						
SLIDE NUMBER	NUMBER OF VESSELS PER VESSEL SIZE (um)					
	4.0-7.5	7.5-30	30-50	50-100	>100	Total
Animal #202						
NMBG 0201	589.00	382.00	30.00	5.00	0.00	1006.00
NMBG 0204	560.00	364.00	18.00	6.00	0.00	948.00
NMBG 0207	618.00	394.00	23.00	7.00	0.00	1042.00
NMBG 0210	755.00	572.00	13.00	3.00	0.00	1343.00
NMBG 0213	739.00	443.00	22.00	6.00	0.00	1210.00
NMBG 0216	734.00	543.00	39.00	8.00	0.00	1324.00
NMBG 0219	845.00	527.00	33.00	8.00	0.00	1413.00
Total	4840.00	3225.00	178.00	43.00	0.00	8286.00
Animal #207						
NMBG 0701	1399.00	1615.00	16.00	4.00	1.00	3035.00
NMBG 0704	1148.00	1907.00	31.00	6.00	1.00	3093.00
NMBG 0707	1270.00	1876.00	29.00	4.00	0.00	3179.00
NMBG 0710	1298.00	1583.00	27.00	10.00	0.00	2918.00
NMBG 0713	1302.00	1783.00	26.00	6.00	0.00	3117.00
NMBG 0716	1376.00	1461.00	30.00	3.00	0.00	2870.00
NMBG 0719	1281.00	1634.00	36.00	4.00	0.00	2955.00
Total	9074.00	11859.00	195.00	37.00	2.00	21167.00
Animal #176						
NMBG 7601	681.00	406.00	30.00	15.00	1.00	1133.00
NMBG 7605	569.00	359.00	17.00	8.00	0.00	953.00
NMBG 7608	604.00	424.00	24.00	9.00	1.00	1062.00
NMBG 7611	537.00	351.00	18.00	7.00	0.00	913.00
NMBG 7614	439.00	318.00	20.00	6.00	0.00	783.00
NMBG 7616	624.00	429.00	25.00	11.00	1.00	1090.00
NMBG 7620	514.00	411.00	30.00	10.00	1.00	966.00
Total	3968.00	2698.00	164.00	66.00	4.00	6900.00

Table 2.6

ISCHAEMIC RIGHT BASAL GANGLIA - 4 HOURS ISCHAEMIA / NO REPERFUSION						
SLIDE NUMBER	NUMBER OF VESSELS PER VESSEL SIZE (um)					
	4.0-7.5	7.5 -30	30-50	50-100	>100	Total
Animal #202						
ISBG 0201	375.00	275.00	22.00	13.00	4.00	689.00
ISBG 0204	300.00	284.00	25.00	11.00	2.00	622.00
ISBG 0208	374.00	314.00	25.00	14.00	2.00	729.00
ISBG 0211	297.00	327.00	15.00	8.00	4.00	651.00
ISBG 0214	307.00	320.00	28.00	9.00	4.00	668.00
ISBG 0217	324.00	324.00	23.00	14.00	3.00	688.00
ISBG 0220	298.00	281.00	27.00	9.00	3.00	618.00
Total	2275.00	2125.00	165.00	78.00	22.00	4665.00
Animal #207						
ISBG 0701	957.00	1833.00	34.00	6.00	0.00	2830.00
ISBG 0704	1032.00	1700.00	26.00	2.00	0.00	2760.00
ISBG 0707	909.00	1881.00	40.00	5.00	1.00	2836.00
ISBG 0710	898.00	1955.00	29.00	8.00	0.00	2890.00
ISBG 0713	895.00	1685.00	31.00	8.00	1.00	2620.00
ISBG 0716	931.00	1667.00	30.00	8.00	3.00	2639.00
ISBG 0719	1024.00	1515.00	18.00	4.00	1.00	2562.00
Total	6646.00	12236.00	208.00	41.00	6.00	19137.00
Animal #176						
ISBG 7601	268.00	147.00	10.00	5.00	0.00	430.00
ISBG 7604	217.00	154.00	19.00	6.00	1.00	397.00
ISBG 7607	258.00	143.00	15.00	5.00	0.00	421.00
ISBG 7611	270.00	163.00	19.00	6.00	1.00	459.00
ISBG 7614	249.00	141.00	29.00	6.00	0.00	425.00
ISBG 7617	256.00	144.00	26.00	4.00	0.00	430.00
ISBG 7620	221.00	143.00	18.00	3.00	1.00	386.00
Total	1739.00	1035.00	136.00	35.00	3.00	2948.00

Table 2.7

Summary data

Percentage reflow for 3 hours ischaemia/1 hour reperfusion and 4 hours ischaemia/no reperfusion for each microvascular size

	4.0-7.5μm	7.5-30μm	30-50μm	50-100μm	>100μm
Animal #					
130	63.7	29.4	31.3	14.9	33.3
142	46.9	64.6	89.7	19.5	57.1
144	28.2	67.7	144	496	1700
107	44.5	16.7	38.6	69.0	125
mean	45.8	44.6	76.0	194	478
standard deviation	12.6	22.1	45.4	18.7	705
202	47.0	65.9	92.7	181	0
207	73.2	103	106	111	300
176	43.8	38.4	82.9	53.0	75
mean	54.7	69.1	94.1	115	188
standard deviation	13.2	26.6	9.7	52.5	112

Figure 2.1

Summary of percentage reflow for each animal at each vessel size - 3 hour ischaemia/1 hour reperfusion

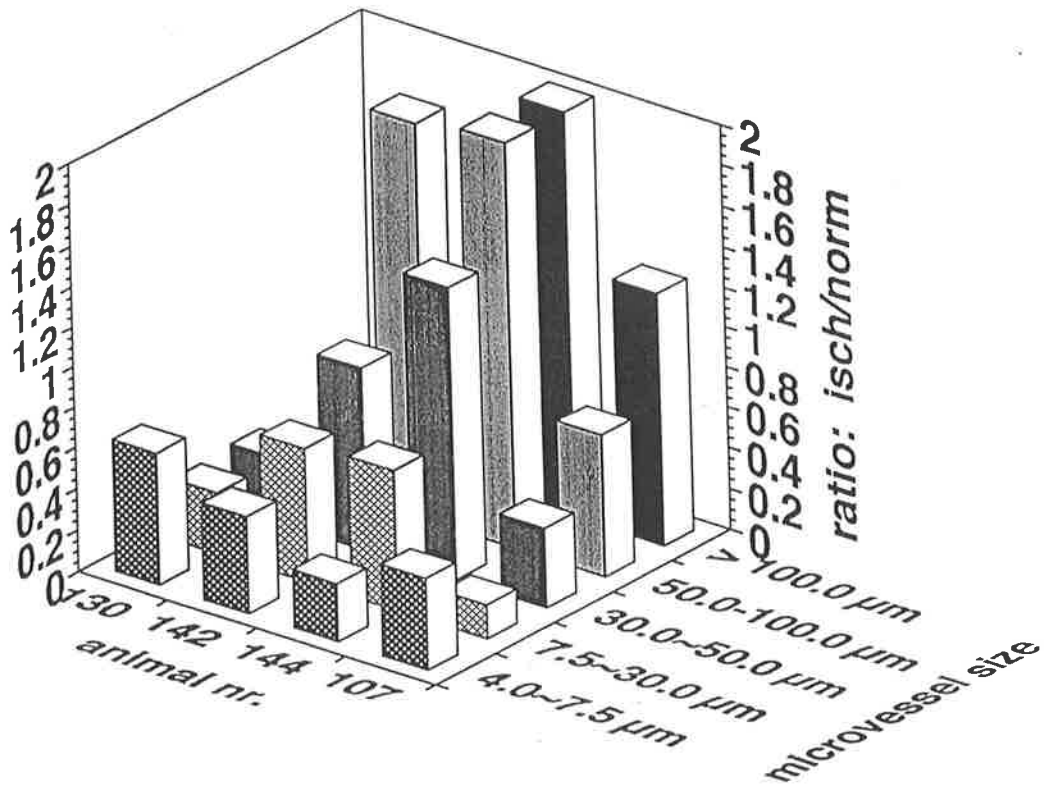


Figure 2.2

Summary of percentage reflow for each animal at each vessel size - 4 hour ischaemia/no reperfusion

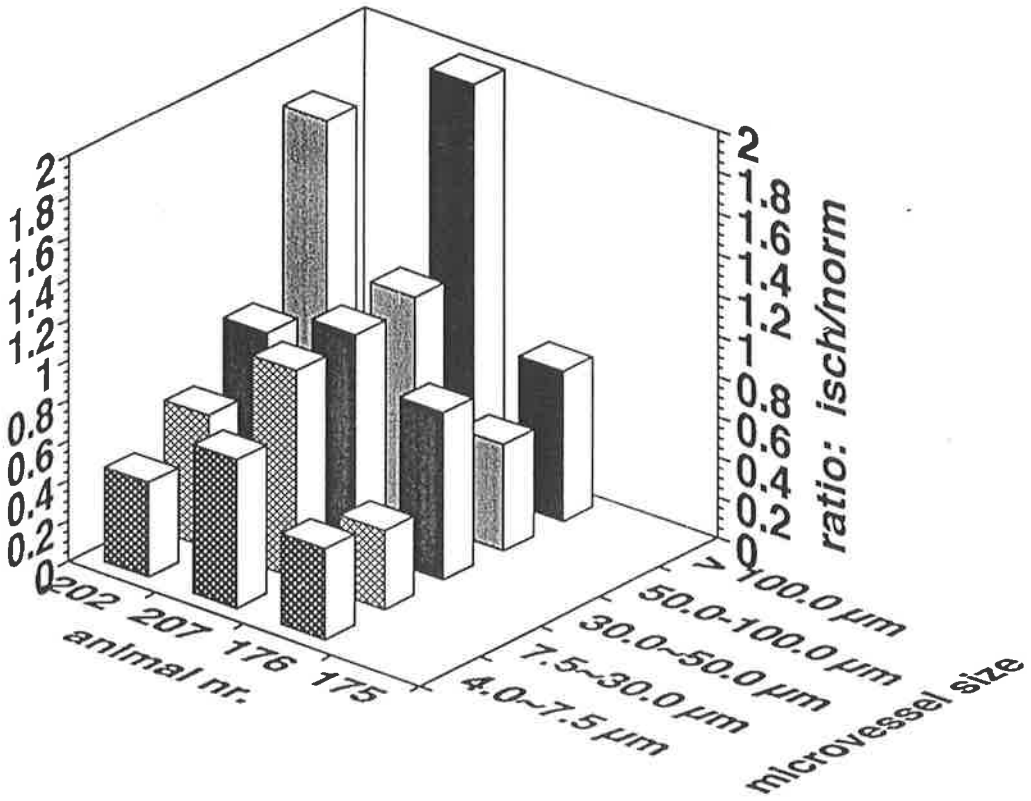
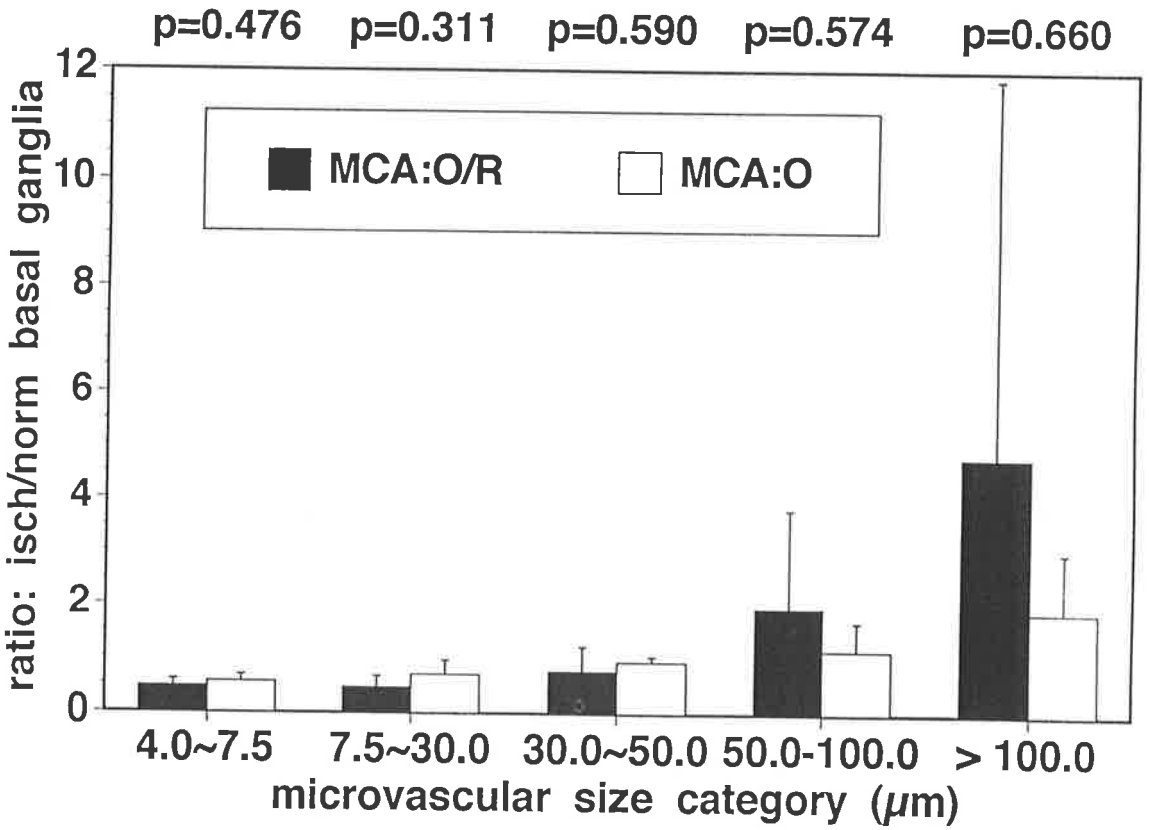


Figure 2.3

Comparison of percentage reflow at each vessel size - 3 hour ischaemia/1 hour reperfusion versus 4 hour ischaemia/no reperfusion



Chapter Three

The effect on the "no-reflow" phenomenon of integrin αIIbβ3 blocking peptide (TP9201) in focal cerebral ischaemia

3.1 Aims

The TP9201 experiments were undertaken to address several questions. The pharmacokinetic and pharmacodynamic studies were undertaken to determine whether the drug was able to be delivered to primates without adverse side effects; to study the pharmacokinetic properties of the drug in primates; and the effect of the drug on template bleeding time.

The experiments in which TP9201 was infused in subjects sustaining focal cerebral ischaemia and reperfusion were undertaken to assess whether this RGD-peptide could modify the "no-reflow" phenomenon. This effect would be mediated by the peptide inhibiting platelet aggregation and hence microvascular occlusions, as discussed in Chapter 1.

3.2 Null Hypotheses

1. The novel RGD peptide TP9201 has no effect on platelet aggregation in primates.
2. TP9201 does not affect microvascular patency during reperfusion following focal cerebral ischaemia in primates.

3.3 Background

Synthetic RGD (Arg, Gly, Asp) -containing peptides can inhibit fibrinogen binding to αIIbβ3 and thus potentially have a useful antithrombotic effect (Plow et al, 1985). The ideal anti-platelet peptide should have the following features:

1. Sufficiently potent for a reasonable dose regime
2. Dose-dependent efficacy
3. Limited bleeding time prolongation at therapeutic doses
4. Short serum half-life

5. No effect on blood pressure, no immune response or anaemia, granulocytopaenia or thrombocytopaenia.

The inhibitory effect of these peptides is calcium-dependent to varying degrees and some of them show increased activity by more than 10-fold in low calcium (Plow et al, 1985; Barker et al, 1992).

A recently-developed synthetic peptide G4120 has been shown to inhibit fibrinogen binding to αIIb β3 (Imura et al, 1992). However, the effective intravenous dose required for *in vivo* inhibition of thrombus formation and *ex vivo* platelet aggregation results in a marked prolongation of bleeding time, suggesting that this peptide may have a therapeutic effect with a concomitant risk of haemorrhagic events.

The αIIb β3 inhibitor used in the present study, TP9201, is a cyclic RGD-containing peptide (acetyl-L-cysteinyl-L-asparaginyl-L-propyl-L-arginyl-glycl-L-α-aspartyl-o-methyl–L-tyrosyl-L-arginyl-L-cysteine-amide, cyclic 1 to 9-sulphide) which was supplied by Telios Pharmaceuticals, Inc (San Diego, California). This has been found to inhibit *ex vivo* platelet aggregation at doses which were not associated with significant prolongation of template bleeding time (Tschopp et al, 1992a).

This agent has been studied in the prevention of rethrombosis after thrombolytic therapy for coronary artery occlusion in dogs in which mean lysis time and total dose of tPA was significantly reduced and long term vessel patency was increased (Tschopp et al, 1992b). A further study demonstrated reduction of platelet deposition on Dacron® vascular grafts in baboons without significantly increased risk of bleeding (Faliakou et al, 1992).

These *in vitro* and *in vivo* results support the effect of TP9201 on inhibiting platelet-platelet interaction and possibly platelet-polymorphonuclear leukocyte interaction. Thus TP9201 may be a beneficial adjuvant agent in cerebral reperfusion by reducing microvascular occlusions, as previous work in this laboratory has shown the presence of platelet aggregation in the microvessels probably within 1 hour and certainly within 4 hours of reperfusion contributing to "no reflow" phenomenon (Okada et al, 1994). Direct platelet-endothelial adhesion has not been documented in this model but this interaction cannot be excluded.

In a recent study of the time-course of platelet and leukocyte infiltration in a rat model of cerebral infarction, Garcia et al (1994) found that platelet aggregates were found mainly in the arterial microcirculation and that this occurred most frequently after 12 hours of

ischaemia. Platelets were only found to occlude 1.6 % of microvessels at 12 hours and 5.0% at 48 hours. Whilst reperfusion was not performed in this study, the low number of occlusions caused by platelets and the late onset of platelet deposition, suggest that the temporal paradigm used in the current TP9201 study (3 hours of ischaemia / 1 hour of reperfusion) may be too brief to demonstrate a significant benefit in microvascular patency.

3.4 Materials and Methods

3.4.1 Preliminary Pharmacokinetic and Pharmacodynamic Studies

Initial pharmaco-kinetic and pharmacodynamic studies were undertaken to determine the optimal dosage of TP9201 to be infused during the focal cerebral ischaemia experiments.

A dose-response study in the 4 baboons was undertaken prior to the cerebral ischaemia/ reperfusion experiments in the same animals. A schematic representation of the experiments conducted is shown in figure 3.1. The awake animals were chaired and 20 mls of blood was drawn (10mls in 1ml citrate and 10mls in 1ml heparin) prior to bolus injection of TP9201 . A template bleeding time was also performed. A bolus of 500 μg/kg of TP9201 was then given and this was followed by an infusion of 12 μg/kg /minute for a period of 4 hours. Six mls of blood were taken and stored in heparin at 1, 2 and 3 hours and 2mls of blood taken and stored in heparin at 4 hours 10 minutes (10 minutes after completion of infusion), 4 hours 30 minutes and 5 hours 30 minutes. Template bleeding times were performed at baseline, 1 hour and 3 hours of infusion.

A full blood count, prothrombin time and activated partial thromboplastin time (APTT) were performed on each blood specimen.

TP9201 plasma concentration was measured by HPLC (high performance liquid chromatography) to determine the peptide pharmacokinetics. ADP and collagen-induced platelet aggregation was used to assess TP9201 pharmacodynamics.

Platelet aggregation inhibitory concentrations (IC_{50} value) in citrated and heparinised plasma were performed at each blood sampling.

Platelet aggregation was performed by spectrophotometric methods using a four-channel aggregometer (Bio Data - PAP-4, Biodata Corporation, Hatboro, PA) which records the

increase in light transmission through a stirred suspension of protein - rich plasma (PRP) at 37°. Aggregation was induced with ADP (30μM) and collagen (10μg/ml) and values expressed as a percentage of aggregation.

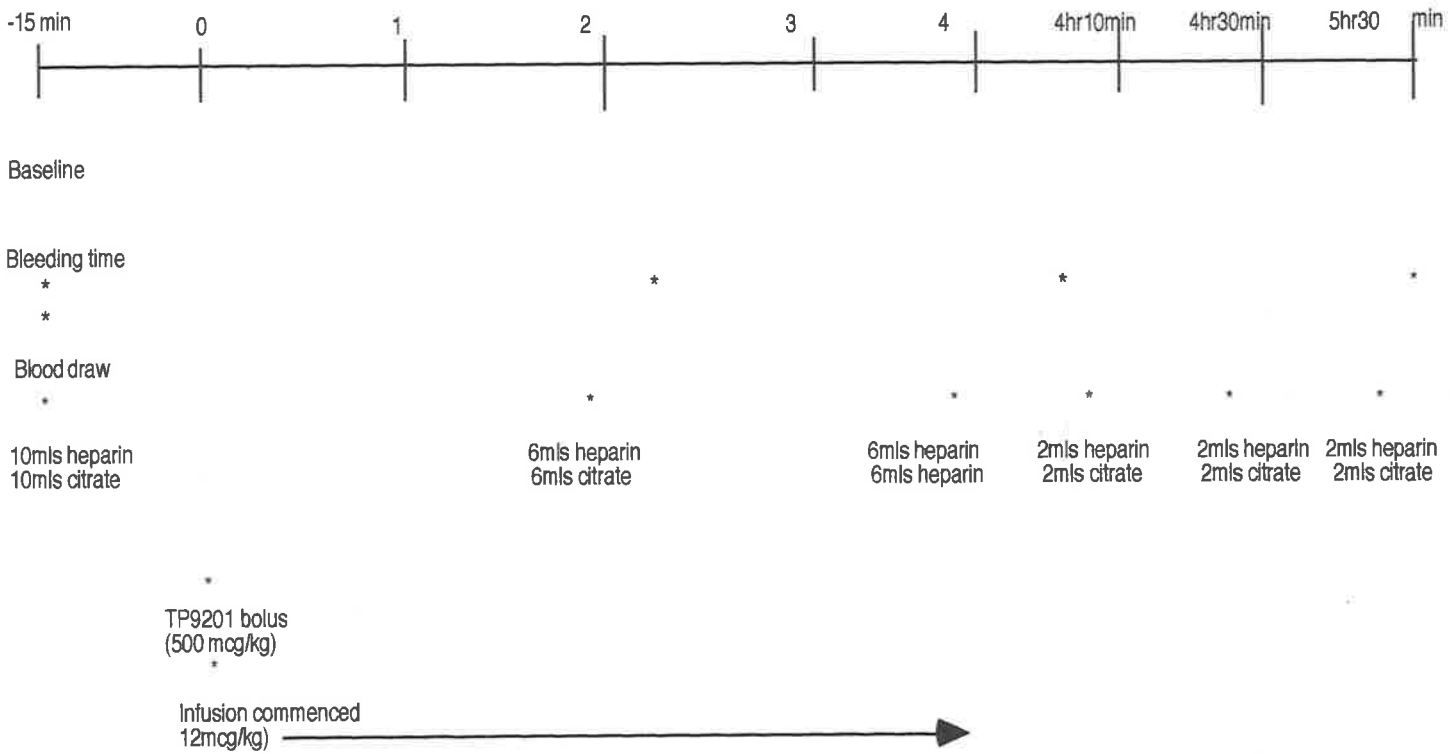


Figure 3.1

TP9201: Pharmacokinetic / Pharmacodynamic Study

3.4.2 "Saline control" study

This study involved 2 of the previously studied subjects (#236, #237) to clarify the cause of leukocytosis found during the TP9201 infusion study. They underwent chairing, baseline template bleeding times and full blood count, and were then given a bolus of saline followed by a 4 hour saline infusion of identical volume as given with the TP9201 infusion study. Full blood counts were taken at identical times to the TP9201 study group.

3.4.3 Focal cerebral ischaemia / reperfusion experiment

Following the TP9201 study, an optimal dose of TP9201 for the focal cerebral ischaemia/reperfusion experiment was calculated by the Telios scientists at a bolus of 675 µg/kg and an infusion rate of 12 µg/kg/minute.

The experimental paradigm followed the previously described MCA occlusion protocol, with placement of the MCA occluding device 7 days prior to the actual experiment.

One of the 4 animals sustained a neurological deficit (N=85/100) during the implantation procedure, but was deemed suitable to continue the experiment. The other 3 animals made an uneventful recovery from the implantation procedure.

A diagrammatic representation of the experiment is shown in Figure 3.2. The chaired subjects underwent baseline template bleeding times and drawing of blood for full blood counts and platelet aggregation studies. The bolus of TP9201 was given intravenously and the infusion commenced. The injectable end of the MCA occluding device was exposed on the scalp by a small incision under local anaesthesia and occlusion was performed with 100-150 µl of fluid into the device.

Following this, all animals sustained a neurological deficit consistent with focal cerebral ischaemia. Neurological scores were measured hourly and blood samples were taken hourly for full blood count and platelet aggregation studies. Bleeding times were measured again at 1 and 3 hours. The infusion was continued until the experiment ended at 4 hours when the subject underwent general anaesthesia and perfusion fixation of the brain with india ink in 4% paraformaldehyde as previously described. No technical problems were encountered during these experiments.

The brain was then removed and underwent a period of 7 days fixation in AFA solution (87% ethanol, 10% of 4% formaldehyde and 3% glacial acetic acid), prior to coronal sectioning at 5 mm intervals.

Visual inspection of the brain was undertaken for macroscopic evidence of decreased perfusion and evidence of haemorrhage. Photographs of coronal sections were taken and diagrammatic representations made. Blocks were then cut of identical sites of both basal ganglia and temporal cortex. The left side (not ischaemic) served as a control. Twenty consecutive sections were cut at 10 μm thickness by Hector Herreira (Department of Cytology, Sharp Hospital, San Diego, California) and stained with basic fuschin/methylene blue.

Blocks were cut from the central basal ganglia, or when haemorrhage was present, from an area of basal ganglia adjacent to the haemorrhage and the identical region on the left side.

The details of the video-imaging system used to measure the number of patent vessels has been given in chapter 2. Twenty sections from the right and twenty from the left basal ganglia were cut and mounted. Each section was approximately 12 x 12 mm in size and 10 μm thick. The imaging system counts 90 fields per section (approximately 5x5 mm). Every third slide is measured (if suitable) at an identical site to ensure that grey matter is measured as exclusively as possible.

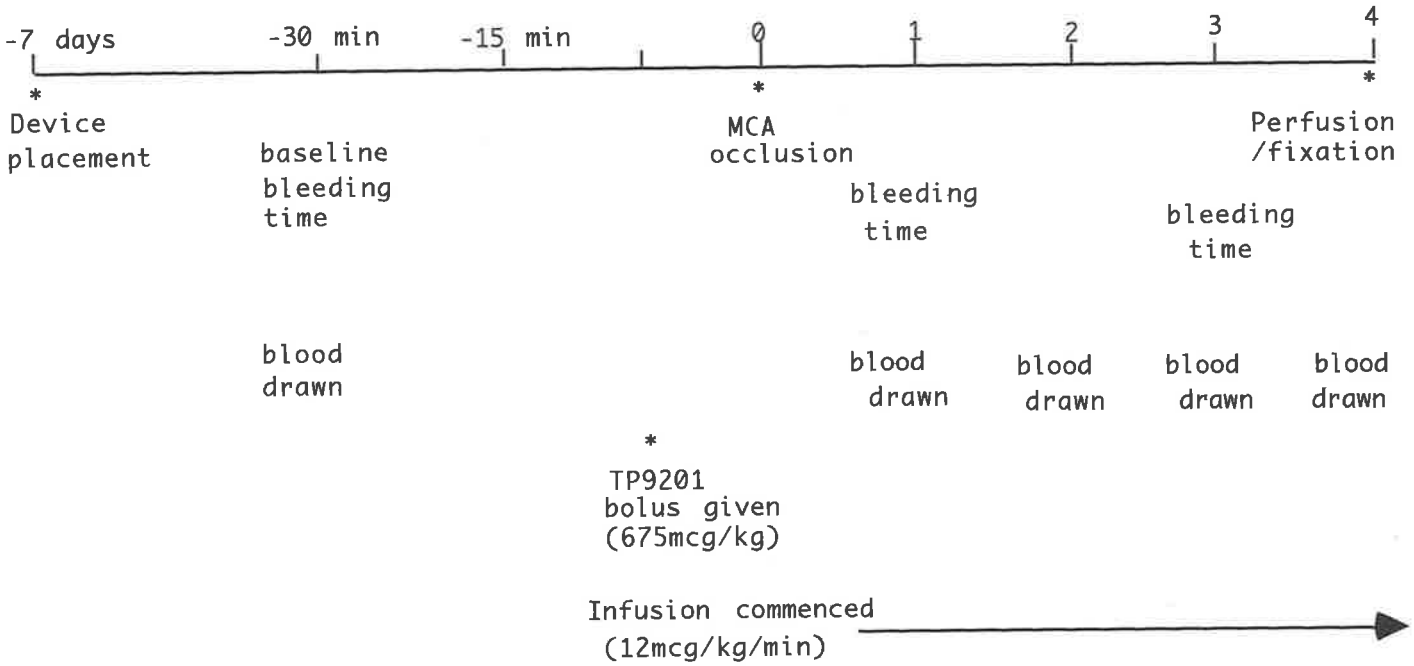


Figure 3.2

TP9201 Focal Ischaemia / Reperfusion Experiment Protocol

3.5 Results

3.5.1 Results of pharmacokinetic and pharmacodynamic study.

Baseline and peri-procedural characteristics of the animals are summarised in Table 3.1.

The subjects suffered no adverse events during this study.

(a) Haematological data:

Basic haematological data are summarised in Table 3.1.

Haemoglobin and haematocrits remained stable throughout the 5hr 30min study period. Baseline haemoglobins were 10.9, 12.8, 13.4, and 13.5 gm/dL (mean 12.7). Platelet counts also remained stable . Baseline values were 215 000, 270 000, 315 000 and 356 000 per μL (mean 290 000).

Baseline white cell counts were 6 400, 9 100, 10 500, and 12 700 per μL (mean 9 700). A marked elevation of white cell count was noticed at 2 hours (27 300, 28 300, 33 000, 34 100 - mean 30 700) which persisted during the 5hour 30minute study period in all animals.

Baseline template bleeding times were 2'30", 2'30", 3'30" and 4 minutes (mean 3'15") in the 4 study animals. Bleeding times were then measured at 2 and 4 hours. In 3 of 4 animals a further bleeding time was performed at 5 hours 30 minutes (i.e. 90 minutes after cessation of TP9201 infusion. An elevation of bleeding times at 2 hours (5'45", 8', 11', 12'30" - mean 9 minutes 20 seconds) and 4 hours (10'30" , 12' , 12'45" , and 15' - mean 12'20") were noted. Ninety minutes after cessation of infusion the bleeding times were 7'15", 8'45", and 9'30" - mean 8 minutes 30 seconds.

(b) Plasma levels of TP9201:

Plasma levels of TP9201 are given in Table 3.2.

Mean plasma levels of TP9201 were 0 at baseline, 2.2 μg/ml at 1 hour, 2.75 μg/ml at 2 hours, 2.6 μg/ml at 3 hours and 2.55 μg/ml at the end of infusion. A rapid decay of plasma TP9201 was noted after cessation of the infusion,with a mean level of 0.8μg/ml 90 minutes after cessation of the infusion. A rapid response of ex-vivo platelet aggregation to this peptide was noted (Table 3.3), both with collagen-heparin and with ADP-heparin.

The IC50 in citrate for the 4 animals was 0.10 ± 0.03 μM and in heparin was 1.67 ± 0.22 μM. The ratio of heparin / citrate of 17.39 was consistent with previous experience in the baboon

dacron fistula model and appears to be due to the changes in calcium concentration rather than the presence of anticoagulant .

3.5.2 Results of "saline control" study

The results of the "saline control" study are summarised in Table 3.4.

Baseline bleeding times were marginally higher than in the TP9201 study (4 and 6 minutes) but were unchanged during the study period.

White cell counts were initially 14,100 and 18,600 per μL (mean 16,400). At 2 hours, the values were elevated to 29.9 and 31.2 (mean 30.6). The leukocytosis persisted during the study period at a slightly lower level, confirming this phenomenon to be a stress reaction rather than an effect of TP9201.

3.5.3 Results of focal cerebral ischaemia / reperfusion with TP9201 infusion

The clinical data and macroscopic findings are summarised in Table 3.5. (The parameters for the subjects not infused with TP9201 are given in Table 2.2)

(1) Haematological data

Subject haemoglobins and platelet counts were stable throughout the experiments. White cell counts were elevated in a similar fashion to that noted previously, commencing at a mean of 11 900, becoming elevated at 1 hour to 24 500 and at 2 hours to 30 800.

In the previously studied 4 MCAO/R animals, mean baseline white cell count was 11 000, at 1 hour of ischaemia was 22 500 and at 3 hours was 25 400.

Similarly, bleeding times became elevated from a mean baseline of 3 minutes to a 1 hour mean of 10 minutes 30 seconds and a 3 hour mean of 10 minutes 30 seconds.

(2) Neurological data

Within 5 minutes of MCA occlusion, a sustained reduction in neurological scores was noted in all subjects in both cohorts. In the TP9201 study group, the scores were 27, 27, 46 and 50 (mean 37.5) and for the previously-studied group subjected to 3 hours ischaemia and 1 hour of reperfusion, the scores were 27, 46, 47 and 76 (mean 49.0). These deficits persisted throughout the four hour study period.

(3) Macroscopic findings

Examination of the previously studied MCA:O/R subject brains demonstrated one specimen with a small petechial haemorrhage only. Right basal ganglia pallor consistent with ischaemia was noted in the brain of each animal. In the TP9201 group, a single animal (#236) sustained right basal ganglia pallor. The remaining 3 animals sustained haemorrhage of varying degrees into the ischaemic basal ganglia (see photographs in Appendix 2). One animal was clearly noted to have midline shift as a consequence of this major haemorrhage.

(4) Microvascular patency

Tables 3.7 and 3.8 detail the number of patent vessels (manifested by those containing carbon tracer) for each given microvascular size in each section. The microvessels of 4.0 to 7.5 μm diameter represent capillaries, 7.5 to 30 μm post-capillary venules and precapillary arterioles, those 30-50 μm, small arterioles and venules, and 50-100 μm large arterioles and venules.

The percentage reflow for each microvascular size grouping was calculated for each animal by calculating the total number of vessels counted on 5 or 7 sections for given vessel size in that animal on the ischaemic (right) side, divided by the total number of vessels counted on identical number of sections for given vessel size in the same animal on left side, expressed as a percentage.

3 hour ischaemia / 1 hour reperfusion cohort (without TP9201)

This cohort also formed the control group in the 4-hour ischaemia study. The results are detailed in Chapter 2, and the microvascular patency data are given in Tables 2.3 and 2.4.

3 hour ischaemia / 1 hour reperfusion cohort (with TP9201)

Percentage reflow data is given in Table 3.9.

Animal #236, which did not sustain an intracerebral bleed, had a mean reflow of 66.6% in the 4-7.5 μm range and 57.0% in the 7.5-30 μm range. In the larger vessel groups, a 51.0% and 47.7% reflow was noted in the 30-50 μm and 50-100 μm range respectively.

The 3 animals sustaining haemorrhage in the basal ganglia had a mean reflow of 130% (149%, 128%, 112%) in the 4 to 7.5 μm range, 158%, (178%, 148%, 148%) in the 7.5 to 30 μm range, 123% (136%, 105%, 129%) in the 30-50 μm range and 2.0 in the >50 μm range.

Overall reflow in each vessel group, compared to the subjects not infused with TP9201 was 114% compared to 46% (for 4-7.5 μm diameter vessels) ($p< 0.01$), 132% compared to 45%

(7.5-30 μm) ($p< 0.01$), and 105% compared to 76% (30-50 μm) ($p< 0.05$), see Figure 3.3.. These were all significant results using a 2 tailed t-test.

3.6 Discussion

The preliminary pharmacokinetic and pharmacodynamic studies confirmed that the novel cyclic RGD peptide, TP9201, could be given safely to baboons with no obvious side effects. Considerable important data was obtained regarding the pharmacokinetics and pharmacodynamics of this agent in an awake primate model.

The two findings of note were firstly, the white count elevation, which has been noted in previous experiments as a "normal" stress reaction to chairing of these animals (del Zoppo et al, 1991). On the basis of the finding of leukocyte elevation during the infusion period, and the potential effect of TP9201 on platelet-PMN interactions, a "saline" control study was performed on 2 of these subjects to assess white cell response to chairing for an identical period of time. The "saline control" study confirmed that the leukocytosis observed during the experiments whilst the animals were chaired was in fact a stress reaction rather than a drug effect.

Secondly, the elevation of bleeding time from a mean of 3 minutes 15 seconds at baseline to 9 minutes 20 seconds at 2 hours; and 12 minutes 20 seconds at 4 hours, was surprising in view of previous experiments utilising this agent, in which no significant elevation in bleeding times were noted (Faliakou, 1992; Tschopp, 1992b). This suggests that the dose used was at the upper end of the therapeutic window - i.e. range between anti-aggregatory effect and elevation of bleeding time, which is evidently broader for this agent than other RGD peptides currently under study.

The subsequent experiments undertaken involved the use of TP9201 in this focal cerebral ischaemia/ reperfusion model. The most significant macroscopic finding in this study was the presence of major intracerebral haemorrhages into the ischaemic basal ganglia in 3 of the 4 subjects. This finding may have several contributory causes. The high perfusion pressure of brain fixative (160-200 mmHg) may be contributary, however the incidence of bleeding has been higher in this study than in previous studies using identical conditions in this model. The high levels of platelet inhibition and elevation of bleeding times resulting from the

TP9201 infusion suggest that the drug may have been given at levels beyond its therapeutic range. This RGD peptide has been shown not to interact with extracellular matrix proteins such as fibronectin and vitronectin (J Tschopp, M Pierschbacher, personal communication). Nonetheless, the presence of ischaemic endothelium and exposed extracellular matrix would presumably predispose to haemorrhage when the function of platelets is so effectively blocked. Continuing work in this laboratory (Hamann et al, 1994, unpublished) has found a decrease in the presence of the basal lamina after periods of reperfusion, which may predispose to haemorrhagic events.

The microvascular patency results from this study are also difficult to interpret. The one animal (#236) which did not sustain an intracerebral haemorrhage was found to have a higher percentage reflow than the control group. In the other 3 animals, and hence the group overall, the percentage reflow in each vessel size for the TP9201-treated animals was greater in the reperfused basal ganglia than in the control basal ganglia. The explanation for this is unclear. Garcia et al (1983) reported early hyperreperfusion of ischaemic brain after 2 hours of occlusion with increased diameter of vessels measured. This persistent post-occlusive hyperaemia may contribute to the expansion and then rupture of microvessels when the duration of ischaemia results in irreversible endothelial damage.

The role of artefact in the patency of vessels in the brain adjacent to haemorrhage is likely to be important. One specimen (#234) clearly had sustained midline shift, and the mass effect of the adjacent haemorrhage would be expected to adversely affect local haemodynamics, possibly compressing microvessels in adjacent and contralateral brain tissue.

The options for future study of this agent are two-fold. The agent could be excluded from further use in this model, or a further study could be conducted using a lower dose of TP9201. A further pharmacokinetic and pharmacodynamic study should be performed to assess the effect of the lower dose on platelet function (partly deducible from the washout data of the present study) and bleeding time. A lower dose which was deemed suitable could then be used to repeat the study.

A study performed concurrently from this laboratory demonstrated that intravascular fibrin "accumulates in a time-dependent manner during focal cerebral ischaemia/reperfusion" (Okada et al, 1994). Intravascular fibrin formation results from the interaction of platelets with leukocytes, and platelet activation. Tissue factor also interacts with fibrinogen to

generate intravascular fibrin. This study showed that although intravascular fibrin was present at all periods of reperfusion (1, 4 and 24 hours) following ischaemia of 2 or 3 hours duration, this accumulation of fibrin was only statistically significant at 24 hours of reperfusion. Thus, the current paradigm of 3 hours ischaemia / 1 hour reperfusion may not be ideal . Rather, a paradigm of 2 hours ischaemia / 8-24 hours reperfusion performed in TP9201-treated subjects and untreated subjects would be more likely to clarify the role of platelet deposition in microvascular occlusions. In addition, the group not treated with TP9201 would potentially be a more suitable group for future reference in studying the "no-reflow" phenomenon.

Table 3.1

TP9201 Pharmacokinetic and Pharmacodynamic Study

	234	236	237	238
Date of procedure	18/10/93	15/10/93	19/10/93	14/10/93
Weight	9.7 kg	8.4 kg	7.9 kg	10.3 kg
Bolus dose	500 µg/kg	500 µg/kg	500 µg/kg	500 µg/kg
Infusion rate	12 µg/kg/min	12 µg/kg/min	12 µg/kg/min	12 µg/kg/min
Baseline:				
Haemoglobin (gm/dL)	12.8	13.5	10.9	13.4
Platelets (per µL)	319,000	356,000	215,000	270,000
WBC (per µL)	9,100	12,700	6,400	10,500
2 hour WBC	28,300	33,000	27,300	34,100
4 hour WBC	22,800	27,200	18,400	24,800
4 hour 30" WBC	24,900	28,500	22,600	29,500
5 hour 30" WBC	26,800	27,200	21,300	30,500
Bleeding time (minutes):				
Baseline	4'	3'30"	2'30"	2'30"
2 hours	12'30"	5'45"	11'	8'
4 hours	10'30"	15'	12'	12'45"
5 hours 30"	-	9'30"	7'15"	8'45"

Table 3.2

Pharmacology of TP9201:

Plasma Levels (μg/ml) during preliminary infusion study

	#234	#236	#237	#238
Duration of infusion (hours)				
0	0	0	0	0
1	2.7	1.54	2.23	2.63
2	2.54	2.51	2.51	3.65
3	2.65	2.28	2.56	3.41
4	2.76	2.35	2.63	2.76
Time after cessation of infusion (minutes)				
10	2.22	1.84	2.17	2.76
30	1.57	1.65	1.37	2.41
90	1.1	1.0	0.69	0.26

Table 3.3

Pharmacology of TP9201:

Ex vivo platelet aggregation in Heparin

(a) Aggregation with collagen (percentage)

Duration of infusion (hours)				
0	100	100	100	100
1	42.2	74.1	64.2	38.0
2	46.1	50.5	65.8	31.7
3	56.9	51.5	55.3	46.6
4	51.0	60.5	47.4	41.2

(b) Aggregation with ADP (percentage)

Duration of infusion (hours)				
0	100	100	100	100
1	30.3	27.8	55.2	25.0
2	22.5	28.5	35.3	17.1
3	29.8	32.9	25.9	26.8
4	29.8	24.7	20.7	22.6

Table 3.4

Saline Infusion Study

	#236	#237
Date	25/10/93	25/10/93
Baseline		
Haemoglobin (gm/dL)	11.8	10.0
Platelets (per μL)	441,000	360,000
WBC (per μL)	10,900	6,900
1 hour WBC	18,600	14,100
2 hours WBC	31,200	29,900
3 hours WBC	26,200	24,700
4 hours WBC	26,900	21,700
Bleeding time:		
Baseline	4'	6'
2 hours	3'30"	5'30"
4 hours	3'30"	6'

Table 3.5

3 Hour Ischaemia/1 Hour reperfusion with TP9201 Infusion (Bolus dose 675 μg/kg, infusion rate 12 μg/kg/min)

	234	236	237	238
Date of procedure	10/11/93	17/11/93	24/11/93	3/11/93
Neurological score				
Baseline	100	100	85	100
1 Hour	27	50	27	42
2 Hours	27	50	27	46
3 Hours	27	50	27	46
Reperfusion	27	50	27	46
Full blood count				
Baseline haemoglobin (gm/dL)	12.3	12.5	11.8	12.2
Platelets (per μL)	423,000	501,000	337,000	4444,000
WBC (per μL)	11,800	13,900	8,000	11,700
1 Hour WBC	28,300	22,600	22,600	24,500
2 Hours WBC	30,800	-	32,500	28,900
3 Hours WBC	24,700	23,300	28,400	21,800
4 Hours WBC	19,000	17,900	20,500	17,500
Bleeding time (minutes)				
Baseline	2'30"	2'30"	3'30"	3'30"
1 Hour	12'30"	5'30"	16'	8'30"
3 Hours	10'30"	11'30"	11'	9'
Macroscopic appearance	Haemorrhage RBG	Small infarct RBG	Haemorrhage RBG	Haemorrhage RBG

Table 3.6

Plasma TP9201 levels during Ischaemia/Reperfusion (μg/ml)

Time (hours)	#234	#236	#237	#238	Mean +/-SD
0	0	0	0	0	0
1	2.70	2.13	2.56	2.42	2.45 +/-0.24
2	2.70	1.92	2.21	2.80	2.41 +/-0.41
3	2.70	1.87	1.89	3.69	2.54 +/-0.86
4	2.47	2.15	1.82	2.76	2.30 +/-0.41

Table 3.7

TP9201 STUDY						
CONTROL (NON-ISCHAEMIC) LEFT BASAL GANGLIA - 3 HOURS ISCHAEMIA / 1 HOUR REPERFUSION						
	NUMBER OF VESSELS PER VESSEL SIZE (um)					
SLIDE NUMBER	4.0-7.5	7.5 -30	30-50	50-100	>100	Total
Animal #236						
NMBG 6T01	1569.00	936.00	42.00	13.00	3.00	2563.00
NMBG 6T04	1451.00	840.00	46.00	14.00	2.00	2353.00
NMBG 6T07	1539.00	893.00	52.00	15.00	1.00	2500.00
NMBG 6T10	1529.00	860.00	38.00	16.00	0.00	2443.00
NMBG 6T13	1460.00	787.00	32.00	18.00	1.00	2298.00
NMBG 6T17	1440.00	768.00	37.00	14.00	0.00	2259.00
NMBG 6T20	1142.00	801.00	57.00	17.00	0.00	2017.00
Total	10130.00	5885.00	304.00	107.00	7.00	16433.00
Animal #237						
NMBG 7T01	992.00	345.00	16.00	7.00	0.00	1360.00
NMBG 7T04	573.00	213.00	11.00	7.00	0.00	804.00
NMBG 7T07	597.00	225.00	15.00	4.00	1.00	842.00
NMBG 7T10	660.00	291.00	19.00	6.00	0.00	976.00
NMBG 7T13	1165.00	393.00	24.00	7.00	0.00	1589.00
NMBG 7T16	895.00	347.00	30.00	8.00	0.00	1280.00
NMBG 7T19	1000.00	330.00	21.00	7.00	0.00	1358.00
Total	5882.00	2144.00	136.00	46.00	1.00	8209.00
Animal #238						
NMBG 8T01	781.00	370.00	27.00	4.00	1.00	1183.00
NMBG 8T05	680.00	322.00	23.00	3.00	1.00	1029.00
NMBG 8T07	629.00	304.00	20.00	4.00	0.00	957.00
NMBG 8T09	846.00	375.00	33.00	5.00	1.00	1260.00
NMBG 8T11	645.00	348.00	21.00	4.00	0.00	1018.00
Total	3581.00	1719.00	124.00	20.00	3.00	5447.00
Animal #234						
nmbg4t01	287.00	116.00	10.00	1.00	0.00	414.00
nmbg4t05	227.00	119.00	8.00	2.00	0.00	356.00
nmbg4t07	337.00	117.00	9.00	1.00	0.00	464.00
nmbg4t11	302.00	126.00	12.00	1.00	0.00	441.00
nmbg4t14	345.00	127.00	12.00	2.00	0.00	486.00
nmbg4t17	632.00	161.00	5.00	5.00	0.00	803.00
nmbg4t19	461.00	140.00	10.00	2.00	0.00	613.00
Total	2591.00	906.00	66.00	14.00	0.00	3577.00

Table 3.8

TP9201 STUDY						
ISCHAEMIC RIGHT BASAL GANGLIA - 3 HOURS ISCHAEMIA / 1 HOUR REPERFUSION						
		NUMBER OF VESSELS PER VESSEL SIZE (um)				
SLIDE NUMBER	4.0-7.5	7.5 -30	30-50	50-100	>100	Total
Animal #236						
ISBG 6T01	908.00	458.00	33.00	7.00	1.00	1407.00
ISBG 6T04	932.00	453.00	19.00	10.00	2.00	1416.00
ISBG 6T07	957.00	485.00	16.00	7.00	1.00	1466.00
ISBG 6T10	1117.00	477.00	18.00	7.00	0.00	1619.00
ISBG 6T13	982.00	492.00	19.00	4.00	2.00	1499.00
ISBG 6T17	920.00	498.00	28.00	10.00	0.00	1456.00
ISBG 6T19	929.00	489.00	21.00	6.00	1.00	1446.00
Total	6745.00	3352.00	154.00	51.00	7.00	10309.00
Animal #237						
ISBG 7T01	721.00	356.00	13.00	8.00	0.00	1098.00
ISBG 7T04	884.00	495.00	20.00	8.00	0.00	1407.00
ISBG 7T07	835.00	373.00	24.00	7.00	0.00	1239.00
ISBG 7T10	1274.00	656.00	31.00	13.00	0.00	1974.00
ISBG 7T13	1275.00	725.00	33.00	7.00	1.00	2041.00
ISBG 7T15	1277.00	614.00	35.00	4.00	0.00	1930.00
ISBG 7T19	1291.00	588.00	29.00	6.00	0.00	1914.00
Total	7557.00	3807.00	185.00	53.00	1.00	11603.00
Animal #238						
ISBG 8T05	1153.00	498.00	17.00	5.00	1.00	1674.00
ISBG 8T07	1174.00	493.00	23.00	13.00	1.00	1704.00
ISBG 8T09	1102.00	495.00	27.00	12.00	1.00	1637.00
ISBG 8T11	717.00	525.00	30.00	8.00	2.00	1282.00
ISBG 8T14	1175.00	526.00	33.00	6.00	1.00	1741.00
Total	5321.00	2537.00	130.00	44.00	6.00	8038.00
Animal #234						
ISBG4t01	454.00	189.00	15.00	3.00	0.00	661.00
ISBG4t02	375.00	188.00	12.00	5.00	0.00	580.00
ISBG4t05	349.00	169.00	10.00	6.00	0.00	534.00
ISBG4t08	374.00	178.00	10.00	7.00	1.00	570.00
ISBG4t10	370.00	205.00	9.00	7.00	0.00	591.00
ISBG4t15	493.00	212.00	13.00	6.00	0.00	724.00
ISBG4t19	499.00	197.00	16.00	3.00	1.00	716.00
Total	2914.00	1338.00	85.00	37.00	2.00	4376.00

Table 3.9

Summary data

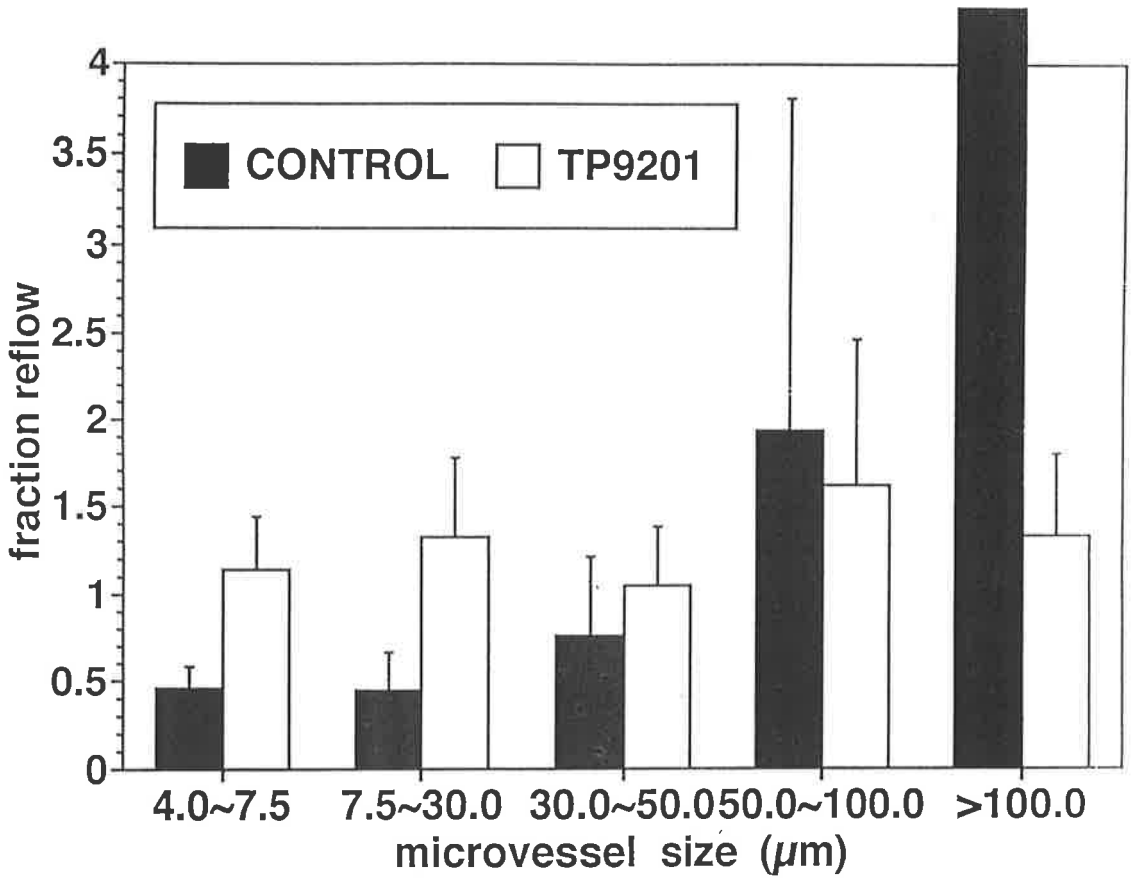
Percentage reflow for control and TP9201 animals for each microvascular size

Animal #	4.0-7.5μm	7.5-30μm	30-50μm	50-100μm	>100μm
CONTROL					
130	63.7	29.4	31.3	14.9	33.3
142	46.9	64.6	89.7	195	57.1
144	28.2	67.7	144	49.7	1700
107	44.5	16.7	38.6	69.0	125
mean	45.8	44.6	76.0	194	479
standard deviation	12.6	22.1	45.4	18.7	706
TP9201					
236	66.6	57.0	51.0	47.7	100
237	128	178	136	115	100
238	149	148	105	220	200
234	112	148	129	264	0
mean	114	132	105	162	133
standard deviation	30.2	45.3	33.5	85.3	47.1

Figure 3.3

Comparison of percentage reflow at each vessel size -

3 hour ischaemia/1 hour reperfusion ± TP9201



Chapter Four

In-situ hybridisation experiments

4.1 Aims

The aims of the in-situ hybridisation experiments were:

(1) to develop the skills required to perform this technique on brain tissues.

(2) to assess the suitability of using blocks of archived tissue (plasmalyte perfused and stored at -70°C) from animals which had sustained varying periods of ischaemia and reperfusion for in-situ hybridisation.

(3) to study the effects of ischaemia and reperfusion on mRNA expression of Tissue factor (TF) and tissue-type plasminogen activator (tPA) in brain subjected to focal ischaemia and reperfusion.

4.2 Null Hypothesis

The null hypothesis of these experiments is that focal cerebral ischaemia and reperfusion has no effect on endothelial expression of TF and tPA mRNA, and hence that the cerebral endothelium does not modulate the coagulation system during ischaemia/ reperfusion.

4.3 Background

4.3.1 Endothelial modulation of coagulation during ischaemia and reperfusion

A detailed discussion of this subject is given in Chapter 1 and will be briefly summarised here.

Vascular endothelium is an anticoagulant and antithrombotic surface. In response to a variety of stimuli, including inflammation and ischaemia, vascular endothelium in cell culture can become procoagulant. Endothelium is capable of generating thrombin, however on "resting" or unactivated endothelium, thrombin is inactivated by antithrombin III or bound by

thrombomodulin. Cytokine and other inflammatory mediators released during hypoxia down-regulate thrombomodulin and up-regulate tissue factor (TF) expression.

Tissue Factor is extensively distributed in the adventia and media of cerebral non-capillary microvessels (del Zoppo et al, 1992). Experiments using a TF-antibody supported the concept that microvascular thrombosis occurs as a result of exposure of blood to sub-endothelial TF in situations of increased endothelial permeability, such as I/R (Thomas et al, 1993). The distribution of TF in the adventitia and media of cerebral vessels, and in astrocytes in the cortex and Bergmann glial cells in the cerebellum suggest that TF forms a "haemostatic envelope" around cerebral vessels, which can activate coagulation when vascular integrity is disrupted (Drake et al, 1989).

In endothelial cell culture, cytokines IL-1 (Nawroth et al, 1986; Bevilacqua et al, 1986), TNF (Nawroth and Stern, 1985; Bevilacqua et al, 1986) and LPS (Colucci et al, 1983) have been shown to up-regulate TF activity. Bevilacqua et al, 1986, also demonstrated an additive effect of two cytokines (TNF and IL-1) on TF activity.

Endothelial cells in vivo have not been shown to express TF under resting conditions or following stimulation with LPS, suggesting different behaviour in vivo than in cell culture (Mackman et al, 1993).

Currently no data are available in vivo regarding regulation of TF mRNA expression during I/R or other cerebral pathological processes, or whether adventitial TF is exposed to circulation clotting factors during I/R secondary to increased vascular permeability.

Tissue-type Plasminogen Activator (tPA) is released continuously and in response to stimuli by vascular endothelium. Gordon et al (1989), demonstrated tPA mRNA in endothelial cells of small venules and the adventitia of larger vessels in primate tissues. The acute release of tPA in response to stimuli such as ischaemia may occur via release of stored tPA or up-regulation of tPA mRNA (Schneiderman et al, 1994). By utilising the techniques of in-situ hybridisation, the role of tPA gene expression during I/R should clarify the role of tPA in modulation of the coagulation system during ischaemia and reperfusion.

4.3.2 In-situ hybridisation background

In situ hybridisation involves the specific binding of a labelled nucleic acid probe to complementary sequences in fixed tissue. The technique can be used to detect RNA, viral DNA or DNA sequences on chromosomes. Initial work was done by Gall and Purdue in 1969 and since then DNA probes were used exclusively until 1990, when Troxler et al (1990) first used a single-strand RNA probe, which has the advantages of greater sensitivity, higher hybridisation efficiency and the absence of competing strands. Critical aspects of this technique are that the target nucleic acid is retained in-situ, is not degraded by nucleases and is accessible for hybridisation with the probe.

Utilising in-situ hybridisation techniques, mRNA activity of a particular protein can give invaluable information in addition to previously available immunohistochemical techniques which detect the amount of protein present. Thus gene expression in tissue sections in reponse to stimuli can be detected in a semi-quantitative manner as well as localising expression to a particular cell type. Complementary immunohistochemical studies localise and semi-quantitate the expression of the actual protein. Northern blot analysis demonstrates specific RNA expression in the tissue as a whole and further complements the other two techniques.

4.4 Materials and Methods

The precise protocols used for the in-situ hybridisation experiments are based on those of Biffo and Tolosano (1992), Wilkinson (1992) and Wilcox (1993) and are given in Appendix 3.

4.4.1 Probe preparation

Two types of single-stranded RNA probes can be used for in-situ hybridisation. Most commonly, the DNA sequence of interest (or part thereof) is cloned into a plasmid vector so that it is adjacent to 2 different RNA polymerase initiation sites at each end of the insert. This allows the synthesis of a sense (negative control strand) and an antisense strand (probe) from

the DNA template. The plasmid is linearised with a restriction enzyme which is selected in such a way that plasmid sequences are not transcribed and the probe synthesized with an RNA polymerase. These probes are typically 250-1000 bases in length. Oligonucleotide probes (20-50 bases in length) can be synthesised when the nucleotide sequence of a particular DNA is known, and can be inserted into a plasmid. They are less sensitive than the above probes but are relatively easy to generate using polymerase chain reaction (PCR) techniques.

The optimal length of RNA probe is probably 400-800 bases, giving optimal specificity and sensitivity. Short probes may give lower signals and high background. Probes longer than 1 kilobase do not readily penetrate the tissue to bind to mRNA. (Probes can be shortened by alkaline hydrolysis).

Labelling of probe

Probes can be labelled with radioactivity or haptens (e.g. biotin) which can be detected by immunohistochemical techniques. Radioactive probes are generally believed to be more sensitive than non-radioactive probes and are far more widely used at present despite the long development times and safety precautions required.

In these experiments, probes were prepared by transcription using ^{35}S-labelled UTP with a specific activity of at least 1000 Ci/mmol (Amersham). (Appendix 3.12).

Probe assessment

0.5µl of probe was suspended in 10 mls of scintillation fluid and the scintillations counted on a computerised scintillation counter. This value could be used to calculate percentage of probe incorporation (the remainder being lost as unincorporated mucleotides). The other 0.5µl of probe was run on a denaturing (RNA gel) to assess probe length and suitability for use in in-situ hybridisation. These gels were run with RNA markers and stained with ethidium bromide, which was usually inadequate for good visualisation. The gel was thus dried in the speedvac between gladwrap and Whatman filter paper. The wafer-like gel was then stored for 2-10 hours sandwiched against an X Ray plate at -70°C and then the plate developed to assess probe length. Having confirmed a successful RNA transcription had occurred, the probe was kept under ethanol at -70°C for up to 4 weeks.

Wilcox has recommended aliquotting desired amounts of the probe to be used over the next 1-2 weeks, to reduce the freeze-thaw cycles performed and hence probe deterioration.

4.4.2 Preparation of tissue for in-situ hybridisation

Tissues for in-situ hybridisation can be prepared for use by sectioning on a cryostat, after embedding in plastic or wax, or tissues or embryos can be fixed and mounted whole.

Effective tissue fixation is essential for in-situ hybridisation and should:

(1) maintain good tissue morphology

(2) avoid degradation and loss of nucleic acids from sections by endogenous nucleases

(3) allow adequate probe penetration (Troxler et al, 1990)

Unfortunately, the conditions for fixation are a compromise, as stronger fixation yields better preservation of tissue morphology but the increased cross-linking (from aldehydes) lowers the accessibility of the target (Wilkinson, 1992). Fixation is generally performed at 0-4°C to inhibit endogenous ribonucleases. The length of fixation depends upon tissue size: for isolated cells, 20-30 minutes is sufficient, for tissues less than 1 cm thick, overnight is sufficient, but for larger tissues (such as brain), perfusion with fixative may be necessary.

The major problem is that RNA stability is low in post-mortem tissues due to the presence of stable, ubiquitous, active enzymes (RNAses) that specifically digest RNA molecules. Whichever technique of fixation is used, tissue RNAses must be rapidly inactivated. This can be performed by

1. rapidly dissecting the tissues out of the animal and freezing them
2. perfusion fixation of the animal by intracardiac injection
3. fixing small blocks of tissue by immersion in an appropriate fixative

Perfusion-fixation (e.g. with 4% paraformaldehyde or 1% glutaraldehyde) is generally recognised as the best method for good presentation of tissue and cellular morphology and optimal RNA retention, although this technique can only be used for laboratory animals under general anaesthesia (Wilcox, 1993)

Freshly-frozen tissue gives good RNA integrity initially, however tissue morphology is not as good. Tissues prepared in this way are usually very small tissue blocks (less than 3x3 mm), which are stored at -80°C, cut at the time of the experiment, mounted on poly-L-lysine slides and immediately fixed with 4% paraformaldehyde (Wilkinson, 1992). The tissues available in our laboratory consist of primate brain (and arteries) which has been pressure-perfused with plasmalyte, rapidly removed, sectioned into blocks of 1 cm x 1 cm x 0.2-0.5 cm and

snap frozen in OCT at -70°C. These tissues are stored at -70°C and cut when required (predominantly for immunohistochemistry) on a cryostat and mounted onto poly-L-lysine slides. They are air dried (to fix to slides) and then fixed with 4% paraformaldehyde for 30 minutes at 4°C. This technique gives good tissue preservation for immunohistochemistry but probably results in considerable RNA deterioration for in-situ hybridisation. Perfusion fixation with plasmalyte followed by 4% paraformaldehyde and then following the above procedure should give optimal results.

4.4.3 Slide Preparation

Slides must be treated ("subbed") to ensure that tissue sections or cells adhere throughout the treatment and washes. The elimination of any exogenous RNAses must also be ensured. Poly-L-lysine (50μg/ml), is widely used and has been mainly utilised in this laboratory (Appendix 3.14)

4.4.4 Prehybridisation

Prehybridisation forms the initial series of treatments in day 1 of the in-situ hybridisation protocol, which is described in Appendix 3.15.

Following mounting the sections on "subbed" slides and treatment with 4% paraformaldehyde in phosphate-buffered saline (PBS), the tissues undergo pretreatment, which is intended to increase the efficiency of hybridisation and decrease non-specific binding. Proteinase K (or a pronase) digestion is used to increase RNA accessibility to the probe. However, this step results in some tissue architecture deterioration (particularly in tissues not fixed by aldehyde perfusion) and thus the duration and concentration of proteinase K used in this step must be carefully titrated. Proteinase K treatment is followed by a refixation step to stop further tissue digestion. Non-specific binding to positively-charged amino groups is prevented by acetylation. Acetic anhydride in triethanolamine is used for this reaction.

Tissues are generally then dehydrated by progressively increasing concentrations of ethanol. The hybridisation buffer (without probe) is usually added to the section (≈ 100 μl/section) and incubated for 1-2 hours.

The tRNA and DNA carriers in the prehybridisation and hybridisation solution compete for non-specific binding sites.

4.4.5 Hybridisation

For successful hybridisation to occur, incubation should be undertaken for 5-16 hours at ≈10-15°C below the melting point of the hybrid. This temperature can be calculated, but usually is ≈58-62°C.

The probe is melted at ≈80°C for 5 minutes and added to hybridisation buffer to give $5x10^5$ - $1x10^6$ counts per minute per section in ≈ 100 µL buffer. Dextran is frequently added to the hybridisation mixture as it has been shown to increase the kinetics of hybridisation by a factor of five (Angerer et al, 1987), and the viscosity of the hybridisation mixture following addition of dextran results in easier and more even application of probe to the section. Dextran in a concentration of 10% was added in later experiments to the hybridisation buffer and a coverslip is applied and the section incubated for 18 hours at 45-50°C, as the sections deteriorated when incubated at higher temperatures in our laboratory.

4.4.6 Post-hybridisation washes

Post-hybridisation washes are designed to remove probe which is non-specifically bound to other RNA sequences. Stringency is the strength of conditions used for post hybridisation washes. It is important to use appropriate stringency conditions (formamide concentration, temperature and salt concentration) to reduce non-specific and partially homologous binding. The appropriate conditions are required to reduce background without compromising specific binding. This can be achieved by washes of appropriate stringency, usually combined with an RNAse treatment, which enzymatically digests non-hybridised molecules (if the concentration of NaCl is greater than 0.3 Molar).

Dithiothreitol (DTT) is a reducing agent which eliminates the formation of disulfide bridges between labelled ^{35}S molecules (Biffo/Tolosano, 1992).

Details of post-hybridisation washes used are given in Appendix 3.16.

4.4.7 Visualisation of signal

The detection of the probe in the tissue sections depends upon whether a radio-labelled or hapten-labelled probe has been utilised. For ^{35}S-labelled probes, a signal can be observed by placing the dried slides in an X Ray box overnight with a fast film sitting on them. This gives useful information regarding presence of signal and specificity of activity (i.e. sense versus antisense signal can be crudely compared). Following this, the slides are dipped in Kodak NTB or Ilford #2 emulsion in a dark room at 45°C (Gowans, 1992). The dipped slides are stored in a sealed box with a dessicator at -70°C and developed 2-8 weeks later (Appendix 3.17 and 3.18).

4.4.8 RNAse free conditions

In-situ hybridisation requires ribonuclease-free conditions both for the preparation of riboprobes and to prevent rapid RNA breakdown by ubiquitous RNAses.

Laboratory procedures undertaken to exclude RNAses include:

(1) Glassware, plasticware etc.

Glassware should be rinsed with diethylpyrocarbonate water (DEPC water 0.1%) and then autoclaved, or baked at 180°C for 4 hours. Sterile one-use plastic ware is also suitable for use. Equipment used for in-situ hybridisation should be kept separate from other laboratory equipment.

(2) Hand contamination

RNAses on laboratory workers hands are a major potential source of RNAses, and thus clean disposable gloves must be worn. For practical reasons, frequent glove changes are usually required.

(3) Solution contamination

All solutions should be made with DEPC-treated millipore water (autoclaved). Molecular biology-grade reagents are used.

4.4.9 Controls for in-situ hybridisation

The use of appropriate controls is an important aspect of the ISH technique. The use of sense and antisense probes are extensively-used controls. Messenger RNA is synthesized from DNA in vivo in 3' to 5' direction thus producing a 'sense' mRNA probe, which will not bind to tissue RNA. An 'antisense' probe is complementary to the mRNA so that it will bind to the tissue mRNA during hybridisation. Therefore the cDNA insert can be cloned in the 5' to 3' direction, or transcribed from the opposite direction to the sense probe, as has been performed during this study. This technique gives sense (negative control) and antisense probes of equal length and G-C concentration, giving an excellent control for the ISH technique.

A further control is the use of a positive control, in which a particular mRNA is known to be abundantly expressed by a particular cell line. Recently, a cDNA insert of von Willebrand Factor (vWF) has been obtained. vWF mRNA is strongly expressed on endothelial cells and thus should give a positive signal on sections hybridised in parallel with the other probe under study. A vWF oligoprobe has been successfully used in other studies (Wilcox et al, 1988) as a positive control. mRNAs which are widely expressed in all or many cell types are not good controls, as positive signals throughout the tissue sections does not give any comparison between positive and negative cells in sections.

Other controls include use of Northern blots to confirm the presence in the tissue under study of the mRNA, and collocalisation of the proteins and mRNA using ISH and immunohistochemistry on serial or the same sections. The mRNA signal should be mostly localised over the cytoplasm, although several published studies have accepted nuclear silver grains as positive and quantitated them by counting (Simons et al, 1993).

Nuclear silver grains has been a problem in several experiments conducted, and may in part be due to using excessive amounts of probe ($> 1 \times 10^6$ counts/section rather than $3\text{-}5 \times 10^5$ counts/section). The decreased wash stringency used in these studies was probably also contributary.

4.5 Results: In-situ hybridisation experiments undertaken

4.5.1 Attempts to perform ISH with full-length TF and tPA probes

Full-length clones of human Tissue Factor (TF) cDNA were pJH9TF and pJH96. These were the gift of JH Morrissey, PhD. Both clones had been inserted in plasmid pGEM7Zf(+) (Promega). The inserts were 1,805 base pairs in length and the plasmid was 3,000 base pairs. E coli strain DH5α had been transformed with the plasmid.

The tissue-type plasminogen activator (tPA) cDNA was the gift of EG Levin, PhD. The full length tPA DNA contains 2530 base pairs, coding for a 562 amino acid polypeptide. The 35 amino acids preceding the mature sequence probably constitute a 20-23 amino acid signal peptide followed by a hydrophilic "lead" sequence of 12-15 amino acids (Pennica et al, 1983). The clone had been previously isolated from a cDNA library constructed in the plasmid vector pBR322 from Bowes melanoma cell mRNA. This was digested with Bgl II, resulting in a 1974 base pair fragment, which encodes the tPA protein plus 200 base pairs of the 3' untranslated end. The fragment was cloned into a BamH-1 site of the plasmid vector pBS (Levine et al, 1983). (However, when subcloning was performed in later experiments, the sequencing suggested that the plasmid vector was in fact pBS KS± with the tPA inserted at the BamH-1 site.

Preparation of DNA templates

Transformed E coli was stored in 50% glycine at -70°C. Cells were picked up by a sterile scraper and incubated in E coli growth medium containing ampicillin overnight in a shaking incubator at 37°C. Plasmid purification was performed using the Promega Miniprep Kit, in which ≈ 1.5-2 mls of medium is used for each preparation. The protocol is described under "plasmid purification" in Appendix 3.2.

Basically, the E coli bacteria are lysed and the plasmid DNA bound to the column resin, which is washed and the plasmid DNA eluted into microcentrifuge tubes at the end of the procedure.

The total plasmid length is 3,000 (plasmid) and 1974 (insert) base pairs (in the case of tPA) or 3000 and 1805 base pairs (in TF). DNA is quite robust (particularly compared to RNA) and was stored in water at -20°C.

Prior to transcription, the plasmid DNA must be linearized adjacent to the insert, so that few plasmid sequences are transcribed. Using 2 restriction enzymes which cut the plasmid adjacent to each end of the insert allows the synthesis of a sense and antisense probe of very similar length and G-C concentration (see "controls" in ISH materials and methods).

Linearised DNA		Restriction Enzymes Used	RNA Polymerase
TF 96	sense	Sma I	SP6
	antisense	BamH-I	T7
TF 9	sense	KPN-1	SP6
	antisense	BstX	T7
tPA	sense	KPN-1	T7
	antisense	Sac-1	T3

The restriction enzymes were obtained from Promega and the protocol for DNA linearisation obtained from Sambrook et al. (1989) (Appendix 3.3). Following incubation for 90-120 minutes at 37°C, a TAE or TBE gel (Appendix 3.7) was used to assess completeness of digestion.

Advice was obtained from Pauline Lee, PhD, who recommeded the use of TBE gels. The TBE or TAE gels are made with ethidium bromide, which intercalates between the DNA bases and allows visualisation under UV light. After photographing the digested DNA alongside DNA markers, which allow the DNA length to be assessed, the linearised DNA is cut out of the agarose gel (only possible with TBE gels) and this small block of gel which contains the desired DNA undergoes the gene-clean protocol (using finely ground glass, "glass milk").

The other value of the DNA gel is that the concentration of DNA can be approximated by comparison of the brightness of the linearised DNA band compared to the markers of known DNA concentration.

Linearisation (digestion) appeared to be incomplete on many occasions for reasons that were not clear, with DNA remaining apparently supercoiled when viewed on the gels.

Several enzymes cut the plasmid with a 3' overhang and thus should have the 3' end "blunted" (or filled in) with addition of free nucleotides and Klenow polymerase. This enzyme was added to the reaction mixture at the end of the linearisation reaction with dNTPs and

incubating for 15 minutes at 30°C (linearisation protocol). The enzymes KPN-1 and Sac-1 required this treatment.

Probe preparation

Following extraction of linearised DNA and "gene-cleaning" as described above, the concentration of DNA was measured by one of two techniques:

1. A small volume was run on a TBE gel with markers, allowing visual assessment of approximate DNA concentration.
2. Spectrophotometric analysis of DNA concentration.

Probe preparation was then undertaken, using the transcription protocol (Appendix 3.12). Concentration of UTP in the final reaction mixture needs to be at least 10μM for good transcription, however Biffo & Tolosano (1992) recommend increased UTP concentration (25 μM) for long probes. Thus, increase volumes of hot UTP must be used and dried down, or "cold" (unlabelled) UTP added. "Cold" UTP was added on several occasions, but did not seem to improve probe synthesis.

Following incubation of the reaction mixture, the DNA template was removed by DNAse digestion, and the RNA was precipitated using phenol-chloroform extraction with ethanol wash. This technique removes unincorporated nucleotides.

Most attempts at RNA preparation were unsuccessful, with no pellet forming in association with the tRNA carrier (seen as a white flocculent precipitate just above granular, darker tRNA carrier). As expected, scintillation counts of the supposed RNA (several microlitres at the bottom of the micropipette) were low, suggesting poor probe preparation.

On several occasions, higher counts were achieved, and an RNA gel dried and exposed to an XR plate overnight suggested the synthesis of a series of incomplete transcripts.

Experiment using TF9 sense and antisense

Sections from animal #218 were used. [3 hour ischaemia/ 1 hour reperfusion, plasmalyte-fixed frozen sections. Right (ischaemic) and left (normal) basal ganglia used]

The in-situ protocol (Appendix 3.15) was used with ≈ 600,000 counts per minute (cpm) per section used. Approximately 75μl of prehybridisation buffer was added, however Dextran was not added to the hybridisation buffer.

TF9+ (with cold nucleotides) was found to have 490,000 cpm/μl and thus

1.25μl of probe was used per section in 75μl buffer.

TF9- was found to have 125,000 cpm/µl and thus

4µl was added per section.

This experiment was repeated using sections from the right and left basal ganglia of animal #203.

A further ISH experiment was undertaken using brain and aortic sections from animal #218.

Problems with these experiments included poor tissue preservation and loss of tissue from slides (see **Materials and Methods**) and minimal non-specific activity on autoradiography. Later development of emulsion confirmed the lack of activity.

Modifications which appeared to be required to obtain a signal on well-preserved tissues were two-fold:

1. Shorter probes were necessary. This could be achieved by synthesis of an oligonucleotide DNA insert (30-50 base pairs) in the DNA core laboratory of Scripps Clinic. A more specific probe, which would be more suitable for mRNA which is not abundant involved subcloning the DNA which was available to obtain probes of between 300 and 1,000 base pairs (preferably 300-600 base pairs).

2. Larger specimens such as brain should probably be perfusion-fixed with an aldehyde, such as 4% paraformaldehyde or glutaraldehyde. This was not undertaken for several months due to animal constraints.

4.5.2 Subcloning of DNA to obtain suitable length DNA inserts for in-situ hybridisation

The technique of DNA subcloning involves:

1. obtaining the DNA sequence of the insert and plasmid

2. generating a restriction map, so that appropriate enzymatic digestion of the DNA can be performed, which would excise the desired fragment and also be used on the new plasmid so that the fragment could be inserted into the opened plasmid at an appropriate restriction site.

3. the DNA insert must be ligated into the plasmid.

4. "competent" E coli cells must be made, so that the plasmid can be inserted and thus replicated.

5. colonies containing the desired plasmid must be selected, grown, Mini-prepped and sequenced prior to use of the insert as a DNA template for mRNA probe preparation.

Sub-cloning of pJH96 - Human Tissue Factor cDNA

Human Tissue Factor (TF) is a 263 amio acid glycoprotein. The pJH96 cDNA insert is 1.66 kilobase (kb) in length and had been cloned between the Sma-I and BamH-I sites on pGEM 7Zf(+) [Promega] . The vector is 2.98 kb in length.

The coding sequence is sited between bases 4-789 in the cDNA. A 3' non-coding sequence is sited from bases 793-1623, followed by a partial poly-A tail.

The DNA sequence (supplied by JH Morrissey) was fed into the MAPSORT programme and a restriction map obtained. An enzyme which would cut out a fragment of suitable size in the coding region was sought.

Restriction enzyme EcoR-I cuts the sequence at 0, 646, 1641 and 1662 giving fragments of 646, 995 and 21 base pairs. This 646 base pair fragment was felt to be suitable. (The EcoR-I site was adjacent to the Sma I site.)

Preparation of DNA fragment for subcloning

Digestion of TF96 plasmid was thus undertaken with EcoR-I for 1.5 hours, run on a TBE gel alongside DNA markers. Figure 4.1 (lane 5) shows the linearised bands of plasmid (2.98 kb), and the 2 shorter TF fragments (995 and 646 base pairs) which travel further down the gel. The 646 base pair fragment was excised under UV light and extracted using the "glass milk" protocol (Appendix 3.4).

The plasmid pGEM7Zf(+) was cut with EcoR-I, and this was also excised from a TBE gel and gene cleaned.

The Tissue Factor insert and pGEM were combined in a molar ratio of approximately 3-5:1 insert:plasmid to facilitate plasmid insertion. The plasmid and insert were heated to 65°C for 2 minutes, allowed to cool and T4 ligase added, which facilitates ligation of insert into plasmid. This mixture was incubated overnighted at 16°C. E coli cell line JM109 were supplied with the pGEM7Zf(+). However, E coli cells NM522 and JM109 cells were made competent using the transformation protocol.

Bacterial transformation

Bacterial transformation is a procedure in which suitable strains of bacteria (E coli XLI Blue for tPA and E coli NM522 for TF) are "transformed" with circular plasmids of DNA containing the desired insert between restriction sites and a sequence for antibiotic resistance. The technique involves obtaining logarithmically growing cells, which are suspended in calcium chloride on ice to generate "competent" cells, which are briefly "heat-shocked" at 37°C to allow plasmid entry from the surrounding buffer. The cells are particularly fragile at this stage.

When grown on an agar plate containing antibiotic (Ampicillin ± tetracycline), only those cells containing the plasmid (and hence DNA coding for antibiotic resistance) will grow. Thus colonies which are present after incubation overnight contain the plasmid of interest. Colonies can then be picked off and grown in bacterial growth medium for use or storage in -70°C (with glycerol). The protocol details are given in "Transformation of bacteria", Appendix 3.10) and based on the method described by Sambrook et al (1989).

Approximately 100ng of ligated plasmid containing TF646 DNA were added to 200 µl of competent cells, incubated on ice for 30 minutes and then "heat-shocked" at 37°C for 5 minutes. 1ml of LB culture medium was added and the mixture incubated for 45 minutes at 37°C. The cells were then spread on plates (LB/Agar/Ampicillin) which had been coated with Isopropyl thio-ß-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGal). IPTG and XGal were used to differentiate colonies in which the plasmid had religated without the TF insert from those containing the insert. Colonies containing the pGEM7Zf(+) plasmid without alteration would remain blue, whereas interruption of the sequence leads to a white colour of the colony.

As a negative control, 100µl of transformed bacteria were spread on two plates. These would not contain the plasmid, which also coded for antibiotic resistance and hence should not grow.

Following this incubation, the colonies all appeared blue, suggesting religation of the plasmid without the TF insert.

The most likely cause of this was that the molar ratio of insert:plasmid in the ligation reaction was too low and thus the experiment was repeated, using a higher ratio of insert DNA:plasmid (7.5:1).

Several further attempts resulted in growth of blue colonies only. Following this TF DNA was extracted with phenol-chloroform, and the ligation repeated. Several pale colonies resulted following this procedure. 14 colonies were picked off and grown up overnight. Minipreps were performed the next day and cells also saved in glycerol at -70°C.

The plasmid DNA was cut with EcoR-I and run on a gel (thus excising the insert). All colonies contained the insert. It appeared possible that a concentration problem with IPTG and XGal may have impaired the contrast between blue and white colonies (on previous attempts at growing colonies).

Sequencing of this DNA in the Core Laboratory of Scripps Clinic was unsuccessful. As a result of this, 1µl of plasmid DNA from one colony was reinserted into competent NM522 cells and grown on 4 plates overnight, to ensure a single colony growth. Several pale colonies and one definitely white colony were picked off the next day, grown up and miniprepped.

The TF insert was present in these colonies after digestion with EcoR-I, and thus the plasmid DNA was sent for sequencing.

The plasmid was cut with EcoRI, Sac-I, ApaI and HindIII (Figures 4.1, 4.2 and 4.3). Digestion with EcoRI resulted in cutting out the 646 bp fragment (seen in lane 2, figure 4.1; lanes 1 and 2 in figure 4.2; and lane 2 in figure 4.3). However, when the plasmid was linearised with Hind III (lane 4, figure 4.3) and Apa I (lane 3 figure 4.2, and lane 4 figure 4.1), plasmid of approximately 4.3 kb. length was obtained, as this band ran close to the fourth band ot the lambda-HindIII marker. (The marker bands are given in Appendix 3.6). This suggested that a double insert of TF646 had been incorporated into the plasmid. This was not initially appreciated. Following realisation of this situation, the restriction map was reviewed to find an enzyme which would cut the insert within the 646 bp region. Hepa I was one of the few enzymes which would do this. Thus the double insert could be cut and religated, thus leaving a single TF646 insert in the plasmid.

Use of the enzyme Hepa I to cut the insert was unsuccessful on several occassions (lane 1, figure 4.3). Ava I was then successfully used to cut the double insert and ligation was performed overnight at 16°C. The ligation mix was then inserted into competent NM522 cells and plated. White colonies were seen and picked for growing up.

At this stage, further discussion had taken place with JH Morrissey, who offered a sub-clone for use.

A 780 base pair subclone (pJH24) was obtained. The cDNA was taken from the coding region and cloned between the Bam H-I and EcoR-I site of pGEM7Zf(+).

	Restriction enzyme	RNA polymerase
TF(pJH24) sense	EcoR-I	SP6
antisense	Bam H-I	T7

This plasmid was successfully inserted into competent NM522 cells. White colonies were picked and individually grown in growth media, Miniprepped and plasmid DNA extracted. The DNA was sequenced successfully (see Appendix 5.1 and 5.2). Digestion and "cold" transcription was successfully performed. Figure 4.4 shows the T7 band (lane 1) and SP6 bands (lane 3 and 4). T3 (lane 2) could evidently also be used for preparing the sense strand. The bright "blobs " at the lower end of each lane is the carrier tRNA.

Subcloning of tPA cDNA

The tPA cDNA used was a 1,974 base pair fragment cloned into a Bam H-I site on PBS (Pennica et al, 1983). This sequence was entered into the MAPPLOT programme and a restriction map obtained. EcoR-I was found to cut the plasmid at 0, 804 and 1279, giving fragments of 804, 475 and 3.8 kb (figure 4.5, lanes 1-4). The 475 bp fragment was felt to be the most suitable for use.

The tPA was digested with EcoR-I and the 475 and 804 bp fragments cut out and gene-cleaned These two fragments were ligated into plasmid pBS, which was the gift of E. Levin PhD. However, when sequencing was performed, the plasmid sequence was actually consistent with plasmid pBSIIKS(+/-). EcoRI cuts this plasmid midway between T3 and T7 at plasmid base pair 707. The plasmid was then inserted into competent cells (as described for TF96), which were plated out overnight.

White colonies were seen after overnight incubation. These white colonies were individually picked off and grown up, a Miniprep was performed on each colony (and 0.5 mls save stored in glycine at -70^{o}C).

The plasmid DNA was then cut with EcoRI to confirm that the insert was cut out of each colony. The DNA was also linearised with HindIII and BamHI (separately). Suitable plasmid DNA was then sent for sequencing using M13 and M13r transcriptase. The 804 bp insert was successfully sequenced, however the desired 475 bp insert was not.

A new batch of plasmid from which the 475 bp insert could be excised by digestion with EcoRI was grown up and was successfully sequenced (see Appendix 5.1 and 5.2).

	linearise with	RNA polymerase
tPA475 sense (+)	HindIII	T7
antisense (-)	BamHI	T3

Preparation of "cold" RNA probes using cold rUTP was performed . The nucleotide was not dehydrated as was performed for "hot" transcription as the concentration of cold rUTP was several-fold higher for cold transcription. The RNA was phenol-chlorophorm extracted, and run on an Ethidium bromide-stained denaturing gel. Bands were seen at approximately 500bp length, consistent with full-length transcripts (figure 4.6 - T7 in lane 1, T3 in lanes 2 and 3, and figure 4.7, with T7 in lane 2 and T3 in lane 1).

Radio-labelled probes were then prepared using the tPA475 bp DNA as a template. Good incorporation was obtained as assessed on activity counts.

[tPA(+) $1.1x10^7$, tPA(-) $1.2x10^7$]

Autoradiography of the dried RNA gels also demonstrated good RNA bands (Figure 4.8)

Later transcriptions showed much better results with T7 than T3, which may have contributed to the high levels of non-specific binding during ISH, since T3 gave the antisense probe.

Advice from colleagues confirmed that T7 was a superior polymerase to T7 or SP6.

4.5.3 Von Willebrand Factor as a Positive Control

The lack of strong positive signals obtained during early ISH experiments had made the assessment of the cause of the problems difficult. Thus a probe which would give strong positive control signals was desirable. Wilcox et al (1988) has used an oligonucleotide von

Willebrand Factor (vWF) probe for this purpose as strong signals are obtained with endothelium.

J.Ware PhD works extensively with vWF and donated a 870 bp insert which was felt would be suitable for use as a DNA template . This fragment coded for amino acids 441-730 in the vWF protein and was inserted into plasmid pBSIIKS(-) between the XhO and Not-I restriction sites.

	Restriction Enzyme	RNA Polymerase
vWF Sense (+)	Bstx with Klenow	T-3
	or Sac-I with Klenow	T-3
vWF Antisense (-)	KPN-I	T-7

The plasmid DNA was linearised and the insert cut out (figure 4.9 - lane 1 contained plasmid and DNA linearisd with KPN-I; lane 3 plasmid linearised with BstX and lane 5 plasmid linearised with Sac-1. The insert was cut out with KPN-1 and Sac-1 in lanes 2,4,6,7,8 and the 870 base fragment is seen). A cold transcription was performed. This was then run on a denaturing (RNA) gel and demonstrated a clear T-7 band, however only a weak T-3 band was seen (figure 4.10). Radio-active probes were then synthesised using new T-3 polymerase. Activity of vWF antisense probewas $8.8x10^6$ cpm/ul and $6x10^6$ for the sense, suggesting reasonable incorporation.

4.5.4 In-situ hybridisation experiment using sub-cloned probes

Using animal number 217 (control cortex and aorta- 1 brain and 1 aortic section per slide).

TPA activity was thought to be potentially a good positive control, however Gordon et al, 1989, found very low levels of tPA mRNA in rhesus monkey aortas, and the tPA activity seen was actually in the adventitial venules adherent to the aorta.

Thus the addition of aortic sections was unlikely to produce useful levels of tPA activity to act as a positive control.

This ISH experiment was performed with the tPA probe and several conditions were varied to try and obtain a good signal as well as better tissue retention and morphology.

(1) Half of the slides were treated with poly-L-lysine and the remainder with chrom-Alum preparation.

(2) Slides were treated with Proteinase K (20 µg/ml) for either 7.5 minutes, as previously used, or for 5 minutes, as the shorter incubation period was likely to improve tissue preservation and architecture.

At this time, Dextran was not used in the Hybridisation buffer. 200 µL of prehybridisation buffer was added, and the slides coverslipped overnight after addition of the probe.

Results : Autoradiography showed minimal activity associated with the sections. Development of emulsion-coated slides after 23 days incubation showed poor retention of brain tissue. The aortic sections were reasonably well-preserved. The aortic sections showed minimal non-specific activity.

ISH experiment undertaken using animal number 174 (control -no I/R, plasmalyte fixed) with 1 section of brain and 1 section of femoral artery or aorta per slide.

tPA was used as the probe (Sense and antisense).

In order to try and improve brain tissue retention, Proteinase K was used for a shorter duration (5 or 7.5 minutes), and sections were cut 10 and 20 µm thick to assess whether thicker sections would demonstrate better tissue architecture.

Results: Tissue preservation was slightly improved. Minimal signal was obtained in the sections, most of which were exposed during autoradiography development.

ISH experiment with major pre-treatment modifications.

The previous ISH experiments had given sparse aortic signals, however the brain sections in particular had been severely digested.

Options considered and used for the next experiment included

1. no Proteinase K treatment
2. A much lower concentration of Proteinase K (1µg/ml cf. 20 µg/ml)
3. Saponin as a pretreatment, as this has been used with good results, allowing tissue penetration by probes whilst maintaining good tissue architecture (Yamawaki et al, 1993). Saponin 0.1% in PBS was used for a 20 minute incubation.

Dextran 10% was added to the hybridisation mix, as this has been shown to improve probe penetration of tissue.

Brain and aorta from animal 174 were used. Ten and 20 μm sections were used.

Results: Autoradiography and emulsion development showed no activity. However, all 3 pretreatment techniques demonstrated much improved tissue preservation.

ISH experiment using paraformaldehyde-fixed brain.

Brain sections of animal 116 (several-year old paraformaldehyde-perfused specimen) were used in addition to sections of animal 174.

The freshly-prepared tPA probes showed discrete bands on autoradiography of the RNA gel and high counts on scintillaton counting, particularly the T-7 probe (Sense probe), figure 4.8.

This experiment was repeated using brain and femoral artery sections from animal 245 (I/R of 3/1 and paraformaldehyde perfused, although probably for an inadequate amount of time due to pump problems during experiment). The second of these 2 procedures underwent hybridisation with tPA and vWF probes.

The post-hybridisation washes were varied , using the 2 protocols of Biffo and Tolosano (1992). One protocol reduces non-specific binding utilising high-stringency washes, and the other mainly by RNAse A treatment. This digests single stranded RNA, thus leaving only RNA bound firmly to tissue mRNA.

Pretreatment was performed with Proteinase K (1μg/ml for 20 minutes) and 10 μm sections were used.

Results: Tissue preservation was much better in the paraformaldehyde-fixed sections.

Minimal signal was seen on the RNAse -treated sections (upper half of figure 4.10).

However, the sections treated with high stringency washes showed strong signals on both autoradiography and emulsion development. (lower half of figure 4.10).

The positive silver grains were mostly localised to the grey matter, and were clustered over the nuclei. The intensity of the signals were indistinguishable in the sense and antisense sections. These findings are consistent with non-specific binding.

The possible causes of high levels of non-specific binding include:

(1) Excessive amounts of probe (e.g. if more than 10^6 counts used per section,which was not the case in this experiment [$5x10^5$ cpm per section])

(2) Contaminated DNA template (with both orientations present). This was considered unlikely as both tPA and vWF hybridised tissues gave the same results.

However, the decision was made to re-sequence both DNA inserts to exclude this possibility. Sequencing excluded the possibility of contaminated DNA.

(3) The use of 50% formamide as a first post-hybridisation wash, and increasing the number of post-hybridisation washes was also considered and adopted for use with half of the sections for the next experiment.

However, the cause of the high level of non-specific binding remained difficult to explain.

In situ hybridisation using protocol modifications to reduce non-specific binding

This experiment was undertaken using sections from animals #245 and 233 (3 hour ischaemia/1 hour reperfusion, adequately pefusion-fixed with 2% paraformaldehyde).

Control cortex and aorta were used. Probes used were tPA and vWF with $3x10^5$ counts added per section. Formamide (50%) was used as first post-hybridisation wash as a variation of the standard protocol in half of the sections .

Results: Autoradiography demonstrated differences in signal intensity obtained for vWF in brain sections of animal 233, suggesting that adequate tissue fixation had not been previously achieved and that endogenous RNAses had contibuted to the difficulties encountered in obtaining signals.

Further experiments were planned using sections from animal 233.

4.6 Discussion

In situ hybridisation is a powerful tool for studying gene mRNA expression in tissue sections. The technique can localise and quantitate mRNA expression, particularly when used in association with immunohistochemistry and, if desired, Northern blot analysis.

This technique involves a series of sophisticated molecular biological techniques as well as meticulous care of tissue sections to prevent loss of the section from the slide, preserve tissue architecture and prevent RNAse digestion of tissue RNA. Whilst the results obtained during these experiments have not obtained definitive results to date, considerable development of the relevant techniques has occurred.

Following the initial failure of the technique with full-length probes (1.9 and 1.8 kilobases), the technique of subcloning these inserts was acquired. Following sequencing of the subcloned inserts, RNA probes were successfully prepared.

Tissue fixation and preparation was also a major problem during the ISH experiments.

The technique of ISH entails multiple washes at various temperatures and in particular a proteinase-K digestion is performed to increase probe access to cellular RNA. These procedures can readily result in loss of tissue from the slide. This was particularly a problem using plasmalyte-perfused frozen sections. The slide preparation technique was modified on several occasions to try and improve tissue adherence.

A more meticulous slide cleaning protocol was adoped, which entailed dipping in 1% 1M HCL in 70% ethanol for 30 minutes followed by dipping in di-ethyl pyrocarbonate (DEPC) water for 30 minutes, followed by dipping in 95% ethanol for a further 30 minutes. The slide racks were placed in an oven at 70°C until dry and then dipped in poly-L-lysine (in Tris) for 30 minutes and dried overnight. Following this, the slides were stored at 4°C with a dessicator for up to 1 month. The use of proprietary-treated slides such as Superfrost Plus was not used during this research period.

Proteinase-K digestion was initially undertaken at a concentration of 20µg/ml and incubated for 10 minutes. However, as the brain tissue used was not paraformaldehyde perfusion-fixed for the majority of the study period, this enzyme resulted in considerable tissue digestion, loss of architecture and tissue loss from the slides. Better results were obtained using the lower concentration of 1µg/ml, however it is unclear whether this lower concentration would affect probe binding adversely.

Saponin has been used to replace proteinase-K and has been reported to maintain good tissue morphology whilst maintaining hybridisation sensitivity (Yamawaki et al, 1993). This was employed during one ISH experiment.

Utilisation of paraformaldehyde perfused tissues resulted in much improved tissue retention on slides and better tissue morphology during the later experiments.

The final series of experiments undertaken utilised adequate probes and well-fixed tissues. The major problem encountered at this stage was non-specific binding of probe. Modifications of technique, including increased stringency of post-hybridisation washes, modification of proteinase K pre-treatment, variation in probe concentration (varying number

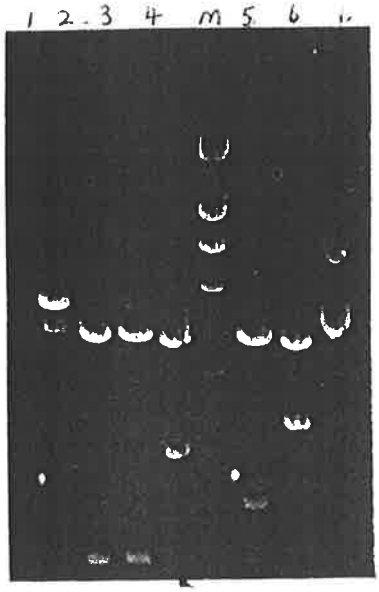
of counts per slide) and addition of formamide in post-hybridisation washes were employed. The DNA inserts used for probe preparation were also re-sequenced to ensure that there was no contamination with reversed inserts.

All of these modifications resulted in minimal improvement in non-specific binding, and thus further input by scientists experienced in this technique appeared essential.

Once it has been successfully developed, this technique provides opportunities to study the behaviour of a large number of relevant genes in this I/R model. Thus sections of preserved tissue can be probed for RNA expression of cytokines, stress proteins, coagulation proteins (such as tPA, PAI-1, TF) and cell adhesion molecules (e.g. ICAM-1, E-selectin). This technique has enormous potential for providing information to understand the mechanisms involved during reperfusion of ischaemic tissues and hence provide insight into possible amelioration of reperfusion injury.

Figure 4.1

Photograph of DNA gel (Tissue factor)



646 base pairs→

Lane 2 and 3 = TF646 cut with EcoRI

Lane 4 = TF646 cut with ApaI and SacI to remove insert (shows 1.3 kb insert)

Lane 5 = TF646 cut with EcoRI (giving 995 and 646 base pair fragments)

M = Lambda/Hind III Marker

Figure 4.2

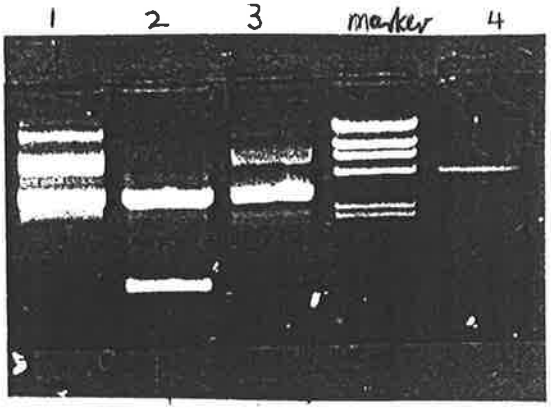
Photograph of DNA gel (Tissue Factor)

Lanes 1 and 2 = TF646 cut with EcoRI

Lane 3 = TF646 linearised with ApaI (shows length of 4.3 kb, suggesting double insert)

Figure 4.3

Photograph of DNA gel (Tissue Factor)



Lane 1 = Plasmid containing TF646 digested with HepaI

Lane 2 = Plasmid containing TF646 digested with EcoRI

Lane 3 = Plasmid undigested

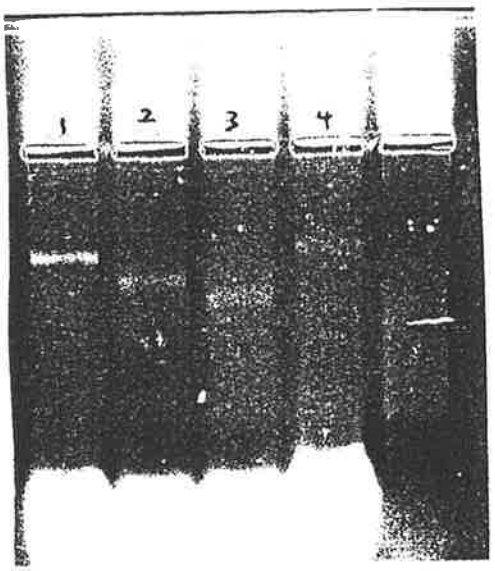
Lane 4 = Plasmid containing TF646 digested with Hind III (length=4.3 kb, suggests 2 inserts)

M = Marker

Figure 4.4

Photograph of RNA gel

("cold" transcription of Tissue Factor Probe PJH24)



Lane 1 = T7 RNA polymerase

Lane 2 = T3 RNA polymerase

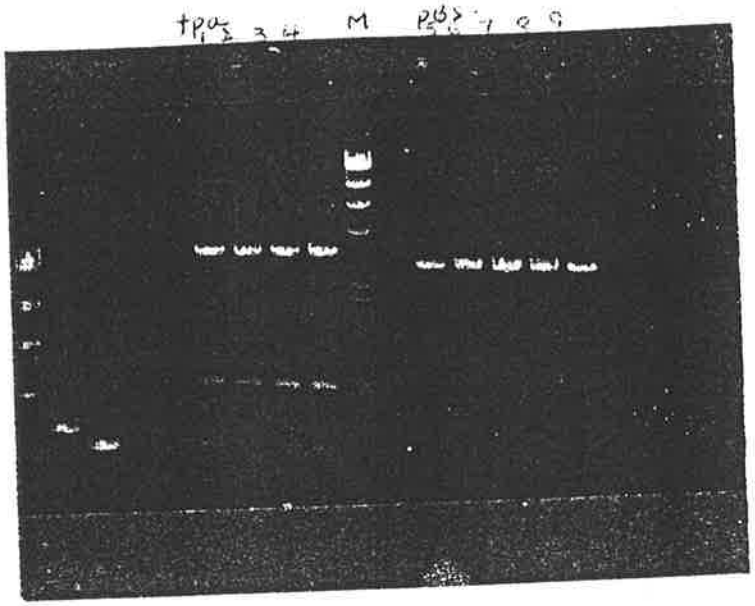
Lane 3 = SP6 RNA polymerase

Lane 4 = SP6 RNA polymerase (different batch)

Figure 4.5

Photograph of DNA gel

(tPA digestion with EcoRI)



Lanes 1-4 = tPA digestion with EcoRI shows 3 fragments:

(i) plasmid 3 kb

(ii) 804 bp fragment

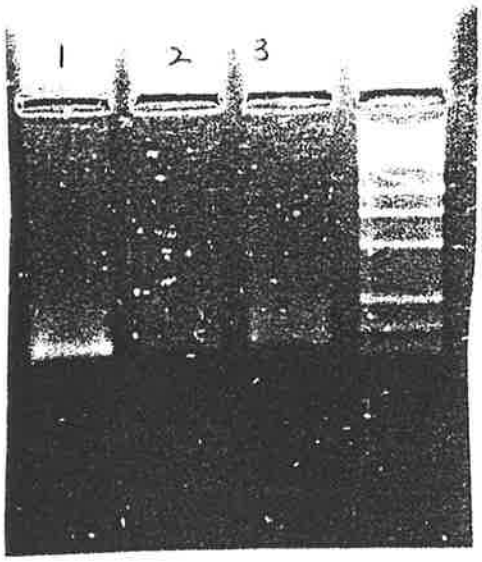
(iii) 475 bp fragment

Lanes 5-9 = Plasmid PBS digestion (3 kb)

Figure 4.6

Photograph of RNA gel

tPA "cold" transcription



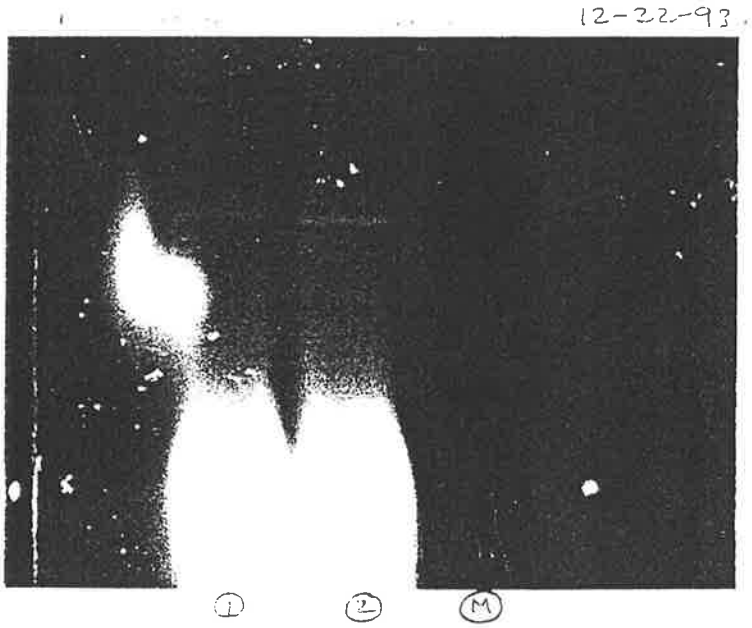
Lane 1 = using T7 RNA polymerase

Lanes 2 and 3 = using T3 RNA polymerase

Figure 4.7

Photograph of RNA gel

tPA "cold" probe preparation



Lane 1= T7 (good band seen at approx. 500 bases)

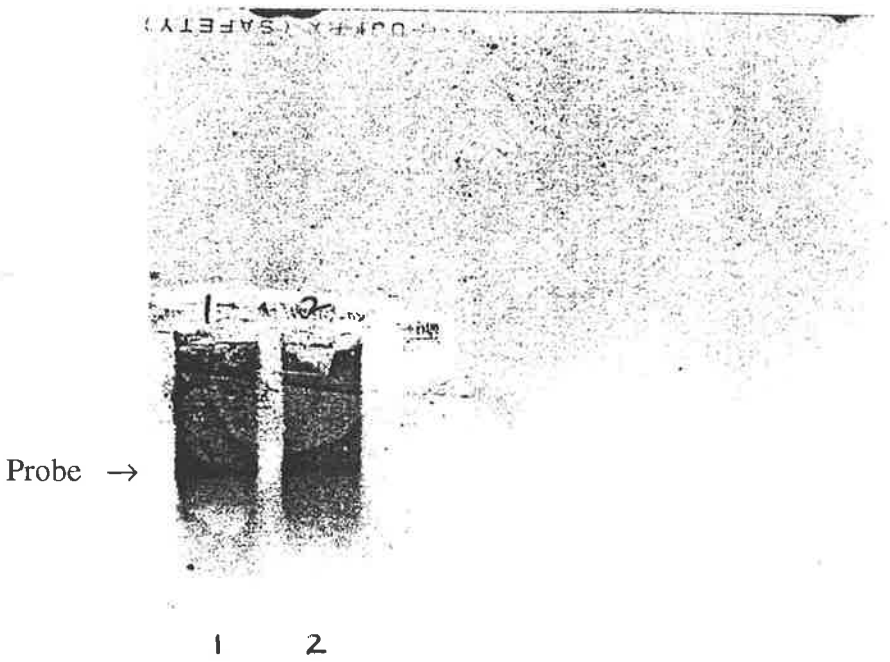
Lane 2 = T3 (good band seen at approx. 500 bases)

(Carrier tRNA at lower ends of Lanes)

Marker not well visualised.

Figure 4.8

Autoradiograph of radioactive tPA probes



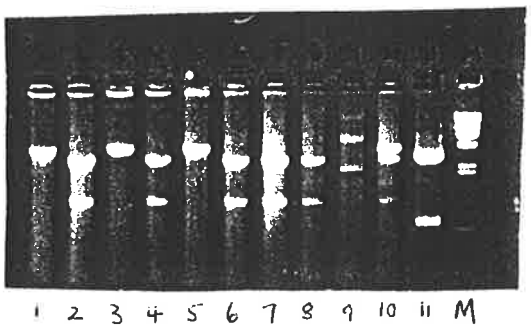
Lane 1 = T7 ("sense" probe)

Lane 2 = T3 ("antisense" probe)

Figure 4.9

Photograph of DNA gel

(vWF digestion)



Lane 1 = Plasmid containing vWF linearised with KPN-I

Lane 3 = Plasmid containing vWF linearised with BstX

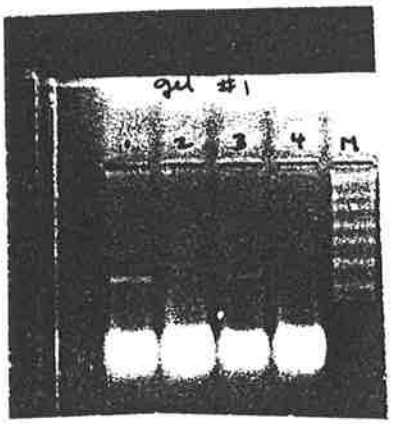
Lane 5 = Plasmid containing vWF linearised with SacI

Lanes 2,4,6,7,8 = vWF insert cut out with KPN-I and SacI

Figure 4.10

Photograph of RNA gel

vWF "cold" transcription



Lane 1 = using T7 RNA polymerase (band seen)

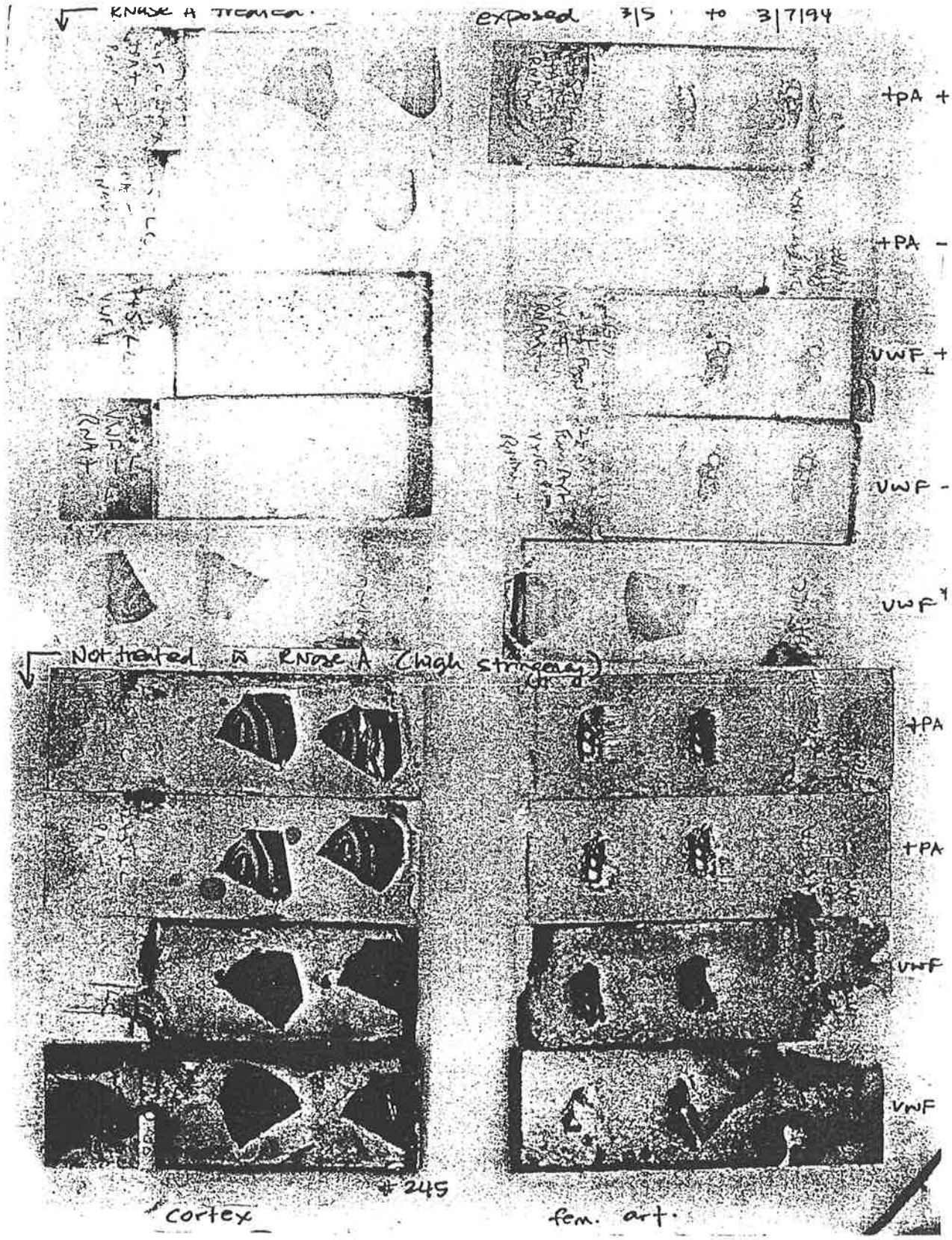
Lane 2 = using T3 RNA polymerase (band not seen)

Lane 3 = using T7 RNA polymerase (band seen)

Lane 4 = using T3 RNA polymerase (band not seen)

Figure 4.11

In-situ hybridisation slide radiography



Chapter Five

Conclusions

5.1 General conclusions

Reperfusion injury is a complex pathophysiological process, which involves a large number of major aetiological factors. These factors have been thoroughly discussed in Chapter 1. The experiments described in this thesis have addressed two major aspects of reperfusion injury following focal cerebral ischaemia. These are the "no-reflow" phenomenon and endothelial modulation of the coagulation system during ischaemia and reperfusion.

The "no-reflow" phenomenon has been extensively studied in this laboratory using a video-imaging system which accurately measures the number and size of microvessels patent in brain sections. The standard paradigm has been 3 hours of ischaemia followed by 1 hour of reperfusion, and this event has been associated with a 30-50% patency of microvessels 4-50 μm in diameter. Previous studies have demonstrated significantly improved patency by infusing a β-2 integrin blocking antibody (to reduce leukocyte adhesion) and an anti-Tissue factor (TF) antibody (Mori et al, 1992; Thomas et al, 1993), confirming the role of leukocyte adhesion and coagulation system activation by exposure of blood elements to adventitial TF in the "no-reflow" phenomenon.

The first two projects undertaken to clarify the "no-reflow" phenomenon further involved a comparison of microvascular patency following 4 hours of middle cerebral artery (MCA) occlusion compared to the standard 3 hours ischaemia followed by 1 hour of reperfusion.

The expectation that there would be significantly lower microvascular patency in the subjects reperfused for 1 hour was not confirmed. This study suggests that 3 hours of ischaemia followed by 1 hour of reperfusion with "unmodified" blood, and 4 hours of ischaemia are periods of injury resulting in irreversible microvascular occlusions in 30-50% of cerebral microvessels. Certainly a study of patency with 3 hours of ischaemia and no reperfusion should be performed to compare with 3 hours ischaemia and 1 hour reperfusion. If the 3 hour ischaemia with no reperfusion also showed 30-50% microvascular occlusions, a more rewarding paradigm would be a 2 hour period of MCA occlusion followed by varying periods of reperfusion (such as 0, 1 or 2, 4 and 24 hours). If a 2 hour period of ischaemia without a

period of reperfusion resulted in a high percentage (80-100%) of microvascular patency on the ischaemic side, this would provide a useful baseline for studying percentage reflow associated with variable periods of reperfusion, and the effects of agents blocking leukocyte adhesion of platelet aggregation.

The second project involved studying the novel cyclic RGD peptide TP9201 in this model. This peptide blocks integrin $\alpha II_b\beta 3$ binding between platelets. Initial pharmacokinetic and pharmacodynamic studies were undertaken to ensure that this peptide could be safely infused into primates (and hence humans), to determine the half-life and drug concentration required to inhibit platelet aggregation.

Following acquisition of these data, the drug was used for a series of 3 hour ischaemia / 1 hour reperfusion experiments. These experiments resulted in 3 of 4 subjects sustaining moderate to large haemorrhages in the reperfused basal ganglia. Microvascular patency was higher in the one subject who did not sustain a haemorrhage than in the control 3 hour ischaemia/1 hour reperfusion group, but greater than 100% on the ischaemic side for each vessel size in each animal sustaining haemorrhage. These results are thus highly likely to be due to artefact. Further studies (see areas for future research) ought to be undertaken prior to use of this drug in human subjects.

The second aspect of reperfusion injury studied was endothelial modulation of the coagulation system during reperfusion following focal cerebral ischaemia. Exposure of cultured endothelial cells to hypoxia increases transmembrane permeability (Shreeniwas et al, 1991), stimulates type 1 Plasminogen activator inhibitor (PAI-1) synthesis (Lynch et al, 1988) and activates coagulation factor X (Ogawa et al, 1990). In contrast with these data, systemic tPA activity has been found to be elevated in severe peripheral ischaemia in humans (Schneiderman et al, 1991).

During reperfusion, intravascular fibrin is generated from fibrinogen by thrombin in the presence of TF (Drake et al, 1989), or activated platelets (Roth, 1992). Thrombin is generated when platelets are activated by exposure to intramural collagen or platelet activating factor (which is generated by ischaemic endothelium). Activated leukocytes can also stimulate thrombin formation (Sugama and Malik, 1992).

Clearly, a time-dependent study of TF, tPA and PAI-1 gene expression by endothelium in-vivo during reperfusion would provide invaluable information which could be used in developing adjuvant strategies in the management of stroke. The technique of in-situ hybridisation is a powerful tool which allows cellular localisation of specific mRNA expression in tissue sections and is ideally suited to studying endothelial behaviour during reperfusion.

Whilst no definitive results have thus far been obtained, considerable expertise has been acquired in molecular techniques which can be readily developed with expert help to obtain useful results.

Successful subcloning of tPA and TF DNA, bacterial transformation and DNA sequencing have been performed. RNA probe preparation and hybridisation with preservation of tissue morphology has been achieved. The final problem to be overcome is non-specific binding for which a series of modifications and checks have not eliminated. Further work on this problem is envisaged.

5.2 Future directions

The results of the microvascular patency studies suggest that several further studies should be considered. The patency data from the 3 hour ischaemia / 1 hour reperfusion versus 4 hour ischaemia support a change of paradigm to a shorter period of ischaemia followed by variable time points of reperfusion such as 0, 1, 4 and 24 hours. A shorter period of ischaemia should result in a percentage reflow of 80-100% when no reperfusion period occurs, progressively decreasing with increased periods of reperfusion as activated leukocytes adhere to post-capillary venules and intravascular fibrin is deposited. Data acquired from these experiments would generate baseline patency rates for studying the effects of adjunctive agents (such as inhibitors of platelet aggregation, antibodies or peptides blocking leukocyte adhesion, e.g. P-selectin antibody or sialyl Lewis^{x}, and thrombolyic agents)

The use of TP9201 could either be abandoned, or re-studied at a lower dose. A further small number of animals (N=2) could be infused for pharmacokinetic and pharmacodynamic studies at a lower dose which inhibits platelet aggregation without a significant increase in bleeding time. Once such a dose has been decided upon, the experiments could be repeated,

probably using a 2 hour ischaemia and an adequate period of reperfusion paradigm (at least 8 hours, as discussed in Chapter 3) if inhibition of intravascular platelet aggregation is to result in significant improvement in microvascular patency.

Further refinement of the in-situ hybridisation technique will allow the use of brain specimen blocks in studying the behaviour of brain endothelium, astrocytes and neurones during reperfusion. In addition to studying mRNA activity of TF and tPA, cellular expression and regulation of cytokine (e.g. interleukins and TNF) and cell adhesion molecule (e.g. E-selectin and ICAM-1) could readily be studied in a time-dependent manner.

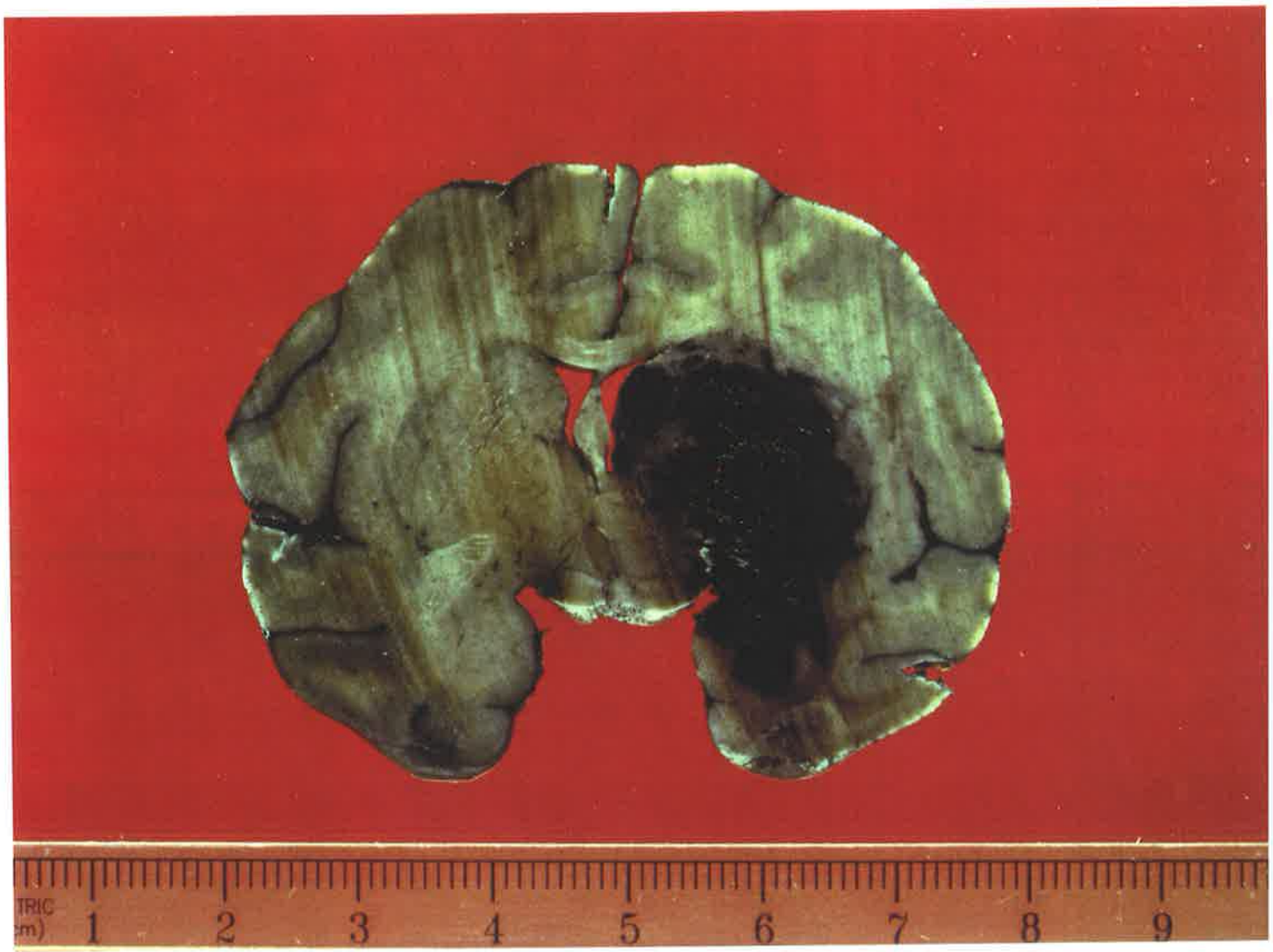
Appendix 1 PRIMATE NEUROLOGICAL EXAMINATION

(based on Spetzler et al, 1980)

	Neurological score
Motor function	
Normal - does not favour either side	70
Normal strength but favours opposite side	55
Mild hemiparesis	25
Severe hemiparesis	10
Behaviour	
Normal aggression	20
Aware of surroundings, moves in response to examiners	15
Aware of surroundings, does not move in response to examiners	5
Coma	1
Death	0
Visual field	
Normal	5
Hemianopia	1
Facial movement	
Normal	5
Paretic	1
Maximum	100

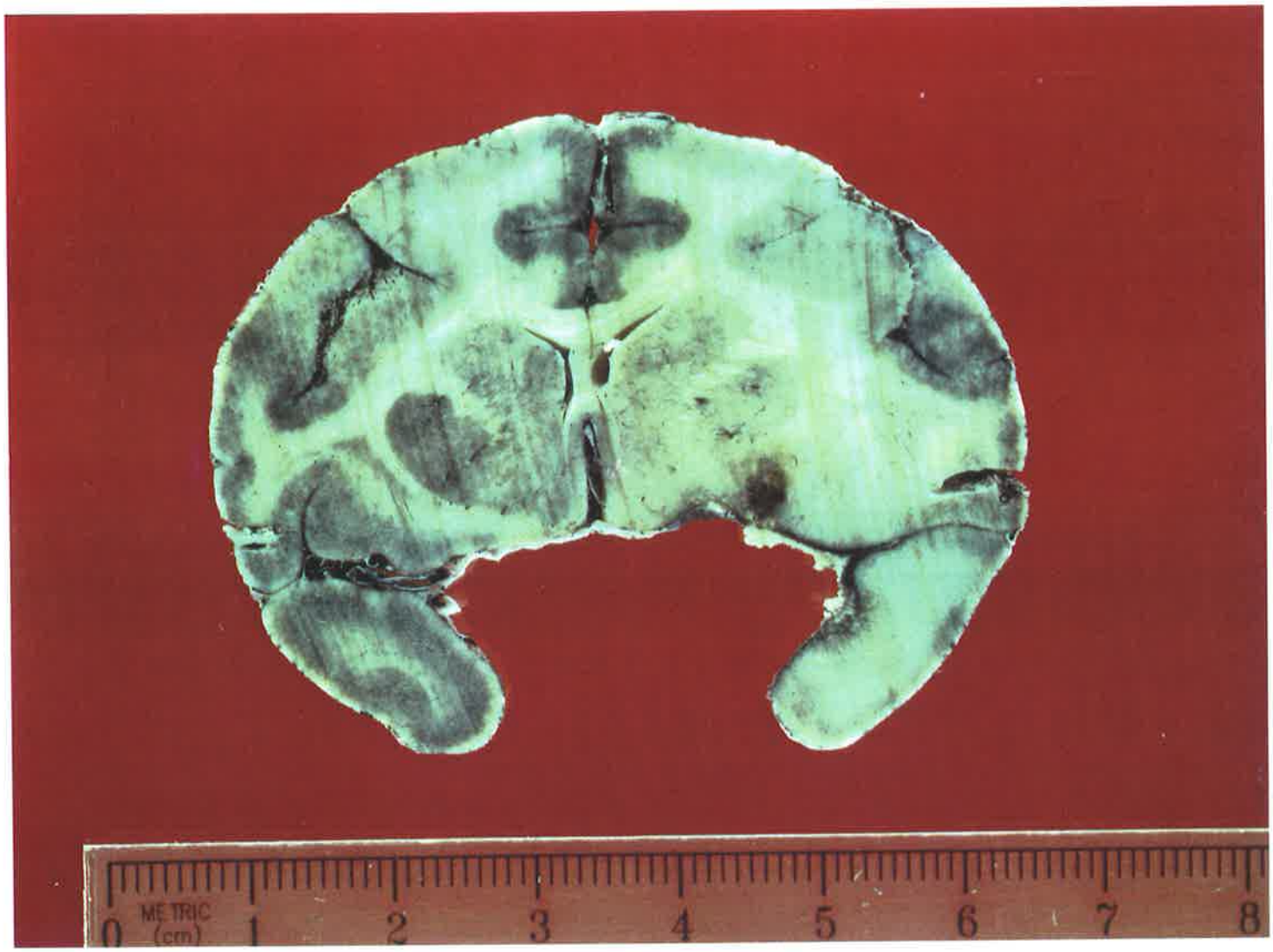
Appendix 2 PHOTOGRAPHS OF CORONAL SECTIONS OF BRAIN

Animal # 175



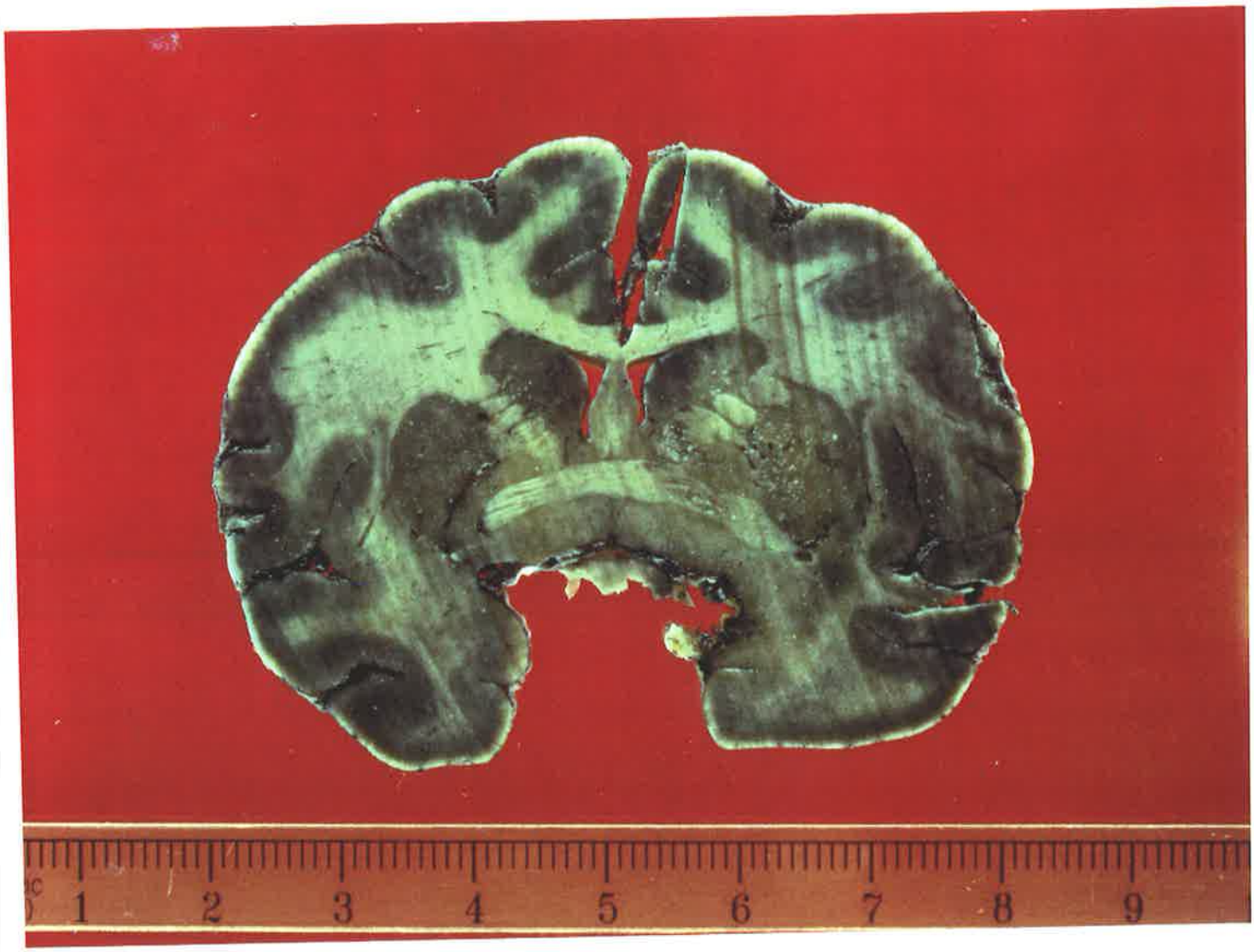
Haemorrhage in right basal ganglia

Animal # 176



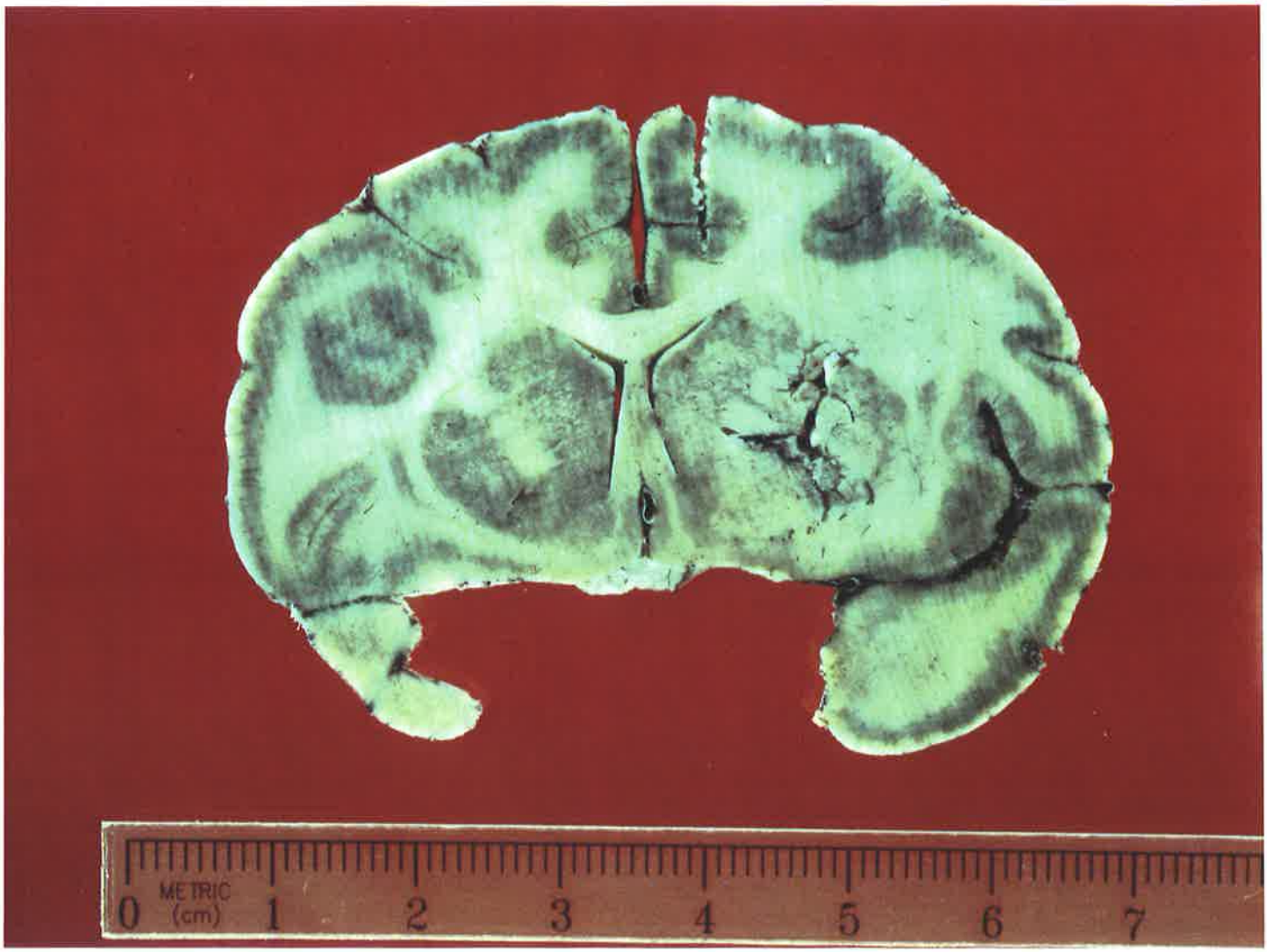
Ischaemic right basal ganglia with small haemorrhage

Animal # 202



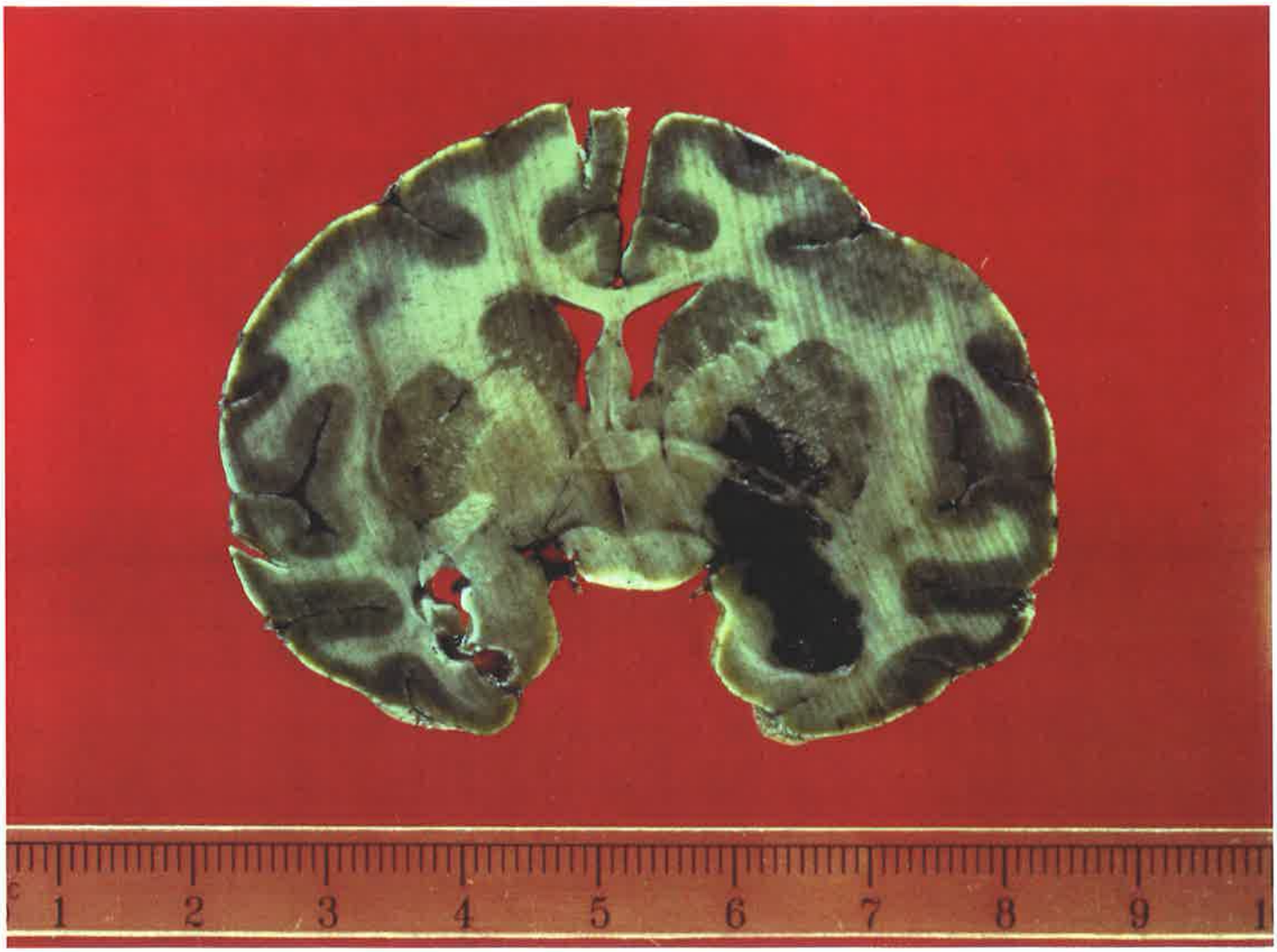
Ischaemic right basal ganglia

Animal # 207



Ischaemic right basal ganglia

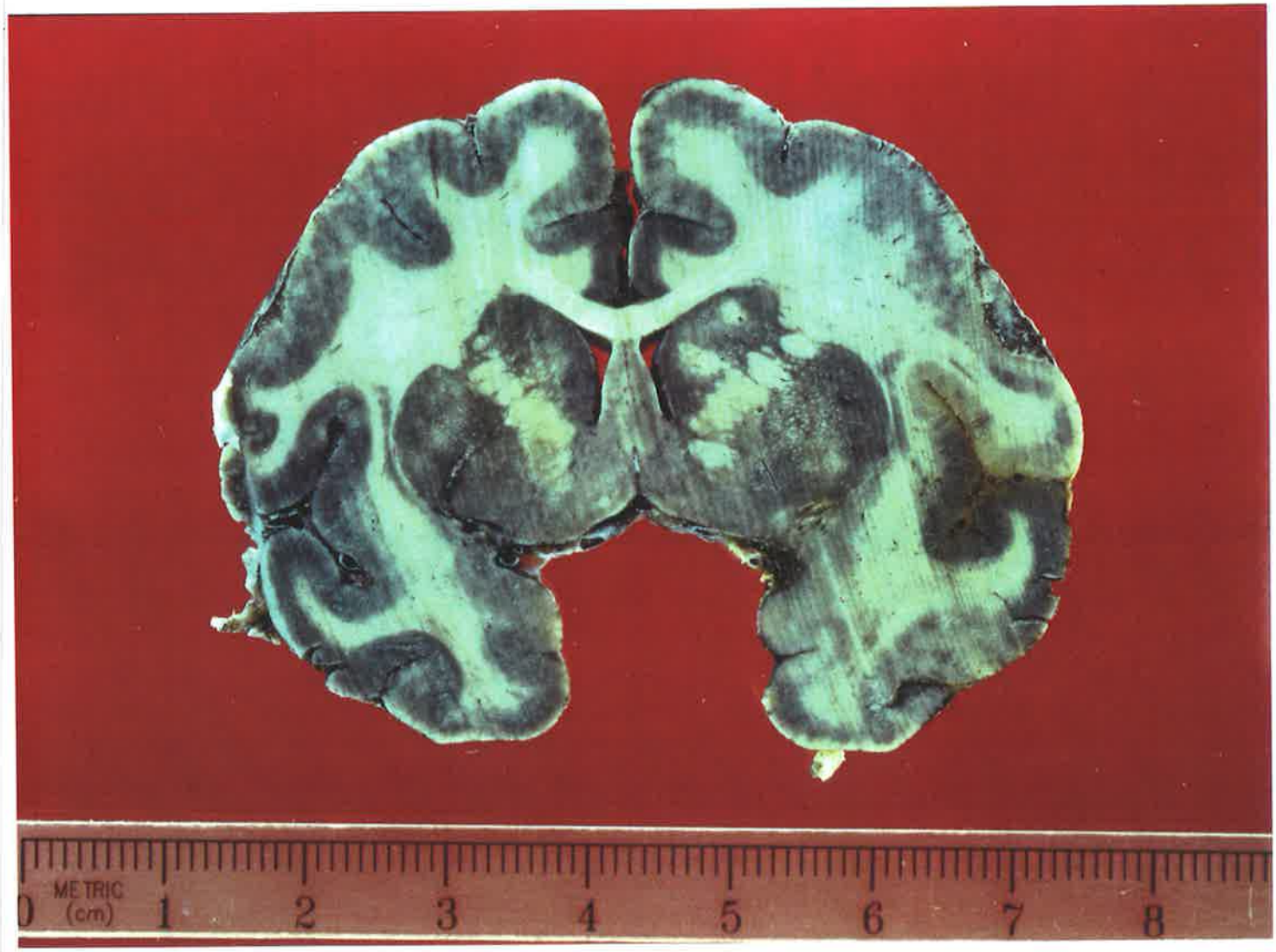
Animal # 234



Haemorrhage in right basal ganglia

note: midline shift

Animal # 236

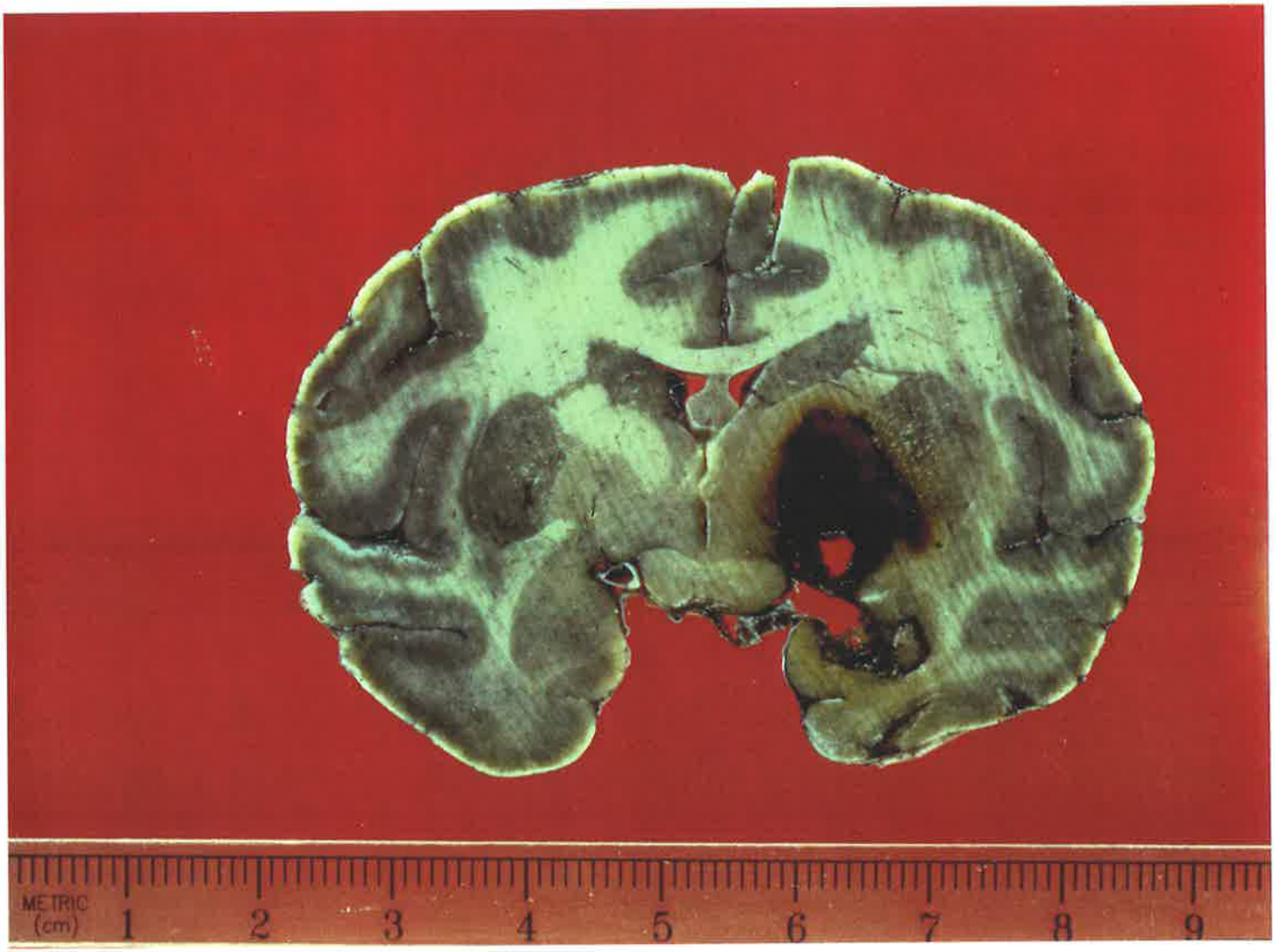


Ischaemic right basal ganglia

Animal # 237

Haemorrhage in right basal ganglia

Animal # 238



Haemorrhage in right basal ganglia

Appendix 3 IN-SITU HYBRIDISATION PROTOCOLS

3.1 Transformation

cDNA of interest in E. coli (stored in 50% glycerol at -70˚C) picked up by scraper and placed in 10 mls of E. coli growth medium (with ampicillin ± tetracycline) and placed in shaker/incubator overnight (37˚C)

ampicillin - 50 µg/ml

tetracycline - 12.5 µg/ml

3.2 Plasmid Purification (Miniprep Kit - Promega)

1. Pellet 1-3 ml of cell solution by centrifugation (3000 RPM for 7 minutes).
2. Resuspend the cell pellet in 200 µl of "cell resuspension solution".
3. Transfer cells to a microcentrifuge tube (1.5 ml).
4. Add 200 µl of "cell lysis solution" and mix by inverting several times. Solution should clear.
5. Add 200 µl "neutralization solution" and mix.
6. Spin in a microcentrifuge at 14,000 for 15 minutes.
7. Decant cleared supernatant to a new microcentrifuge tube, and discard precipitate.
8. For each miniprep Add 1 ml of "Magic Minipreps DNA Purification Resin" to the supernatant from step 7 and mix by inverting the tube.
9. For each miniprep, use one Magic Minipreps mini-column. Attach a 3 ml disposable syringe barrel to the luer-lock extension of each mini-column.
10. Decant the Magic Minipreps DNA Purification Resin containing the bound DNA into the syringe barrel. Insert the syringe plunger and gently push the slurry into the mini-column.
11. Wash the mini-column with 2 ml Column Wash solution by removing the mini-column from the syringe and taking up the solution in the syringe. Reattach the syringe to the mini-column and gently push the Column Wash Solution through the mini-column with the syringe plunger.

Important: The Column Wash Solution is **NOT** supplied with ethanol. Prior to use, sufficient absolute ethanol must be added to the Column Wash Solution to bring the final concentration to 50% (v/v).

12. Transfer the mini-column to a microcentrifuge tube, and place in a microcentrifuge. Spin the mini-column for 20 seconds to dry the resin.

13. Transfer the mini-column to a new microcentrifuge tube.

14. To elute the plasmid DNA, add to the mini-column 50 µl of DEPC treated water or TE buffer heated to 65-70°C.

15. Spin the microcentrifuge tube containing the mini-column for 20 seconds in a microcentrifuge. Remove and discard the mini-column store. Plasmid DNA in a microcentrifuge tube -20°C. Each Magic Minipreps DNA isolation will yield up to 10 µg of plasmid DNA.

NOTE: The yield of plasmid DNA is dependent on the plasmid copy number. The plasmid DNA is ready for immediate use in any molecular biology procedure.

3.3 Linearization of DNA

plasmid DNA	1.5 µg
10 x restriction buffer	2 µl (if total vol 20 µl)
Appropriate Restriction Enzyme	15-25 units (≈ 2 µl) (no more than 10% of total volume)
M.B. H_2O	to 20 µl final volume

Incubate at 37°C for 2 hours.

Add 6x marker. Heat to 56°C for 2 minutes.

Run TAE or TBE gel approximately 50 volts for 2-3 hours

Blunting 3' end of cDNA (if necessary)

Heat DNA to 55°C for 5 minutes.

Add Klenow 1 unit/1 µg DNA and 0.5 mM each dNTP/ µg DNA.

Incubate at 30° C for 15 minutes, heat inactivate 75° for 10 minutes.

Run gel with marker to assess completeness of digestion.

3.4 Gene Clean Protocol (DNA purification using glass milk)

1. Run TBE gel for 45-60 mins.
2. Turn on water bath to 56°C. Defrost glass milk.
3. Cut DNA out of gel under UV. Do it quickly (take <20 seconds so that DNA not damaged).
4. Weigh gel (must be <0.4 g.)
5. Add 2 µl NaI for each mg. agarose.
6. Melt agarose in waterbath ≈5 minutes (discard NaI if it is yellow).
7. Add 5 µl glass beads (1 µg/ 1 µg DNA, but a minimum of 5 µl)
8. Ice for 5 minutes.
9. Spin 13K RPM 30 sec.
10. Discard supernatant.
11. Wash pellet 3x with cold NEW SOLUTION (200 µL) (10-50x vol). Resuspend pellet, spin 10 seconds, discard supernatant, repeat.
12. Do one dry spin.
13. Add 10 µl Depc water, mix with pipette tip.
14. Spin 20 sec 13K RPM.
15. Aspirate supernatant (DNA in Supernatant).

NaI Solution

90.8 g NaI
1.5 g Na Sulfite
100 ml H_2O
Filter through Nalgene, 0.2 µm filter
Add 0.5 g Na Sulfite to saturate solution
Store in dark at 4°C (foil covered)

NEW Solution

250 ml ethanol
219 ml H_2O
10 ml IM Tris, pH 7.4
1 ml 0.5 M EDTA
20 ml 2.5 M NaCl
500 ml store -20°C

3.5 DNA Agarose Gel

1% TAE gel: 60ml 1x TAE (hot plate setting = 4)
0.6g agarose
Running buffer:
500ml 1x TAE
30µL Ethidium Bromide

Per Well: 2µL DNA
1µL 6x Dye
3µL DEPC water

Preheat DNA samples at 65°C (2 min).
Spin 3K RPM 1 sec.
Run at 50V for 1-2 hours.
Take photo. Set camera on 11-1.
Maximum well volume: 10µL 30 well comb
60µL 10 well comb

3.6 Marker mix for DNA gels (Lambda/Hind III)

(use 8µL per well)
4µL 2.5M NaCl
10µL 1M Tris (pH 7.5)
2µL 0.5M EDTA
100µL DNA
167µL 6x Ficoll dye
717µL DEPC water
1 ml (2 x 500µL)

The lambda -Hind III Markers consist of 8 fragments of the following sizes (in base pairs):

23,130
9,416
6,557
4,361
2,322
2,027
564
125

3.7 Agents used for DNA gels

6x Ficoll Dye

0.25%	Bromophenol Blue	1. increases sample density
		2. add colour, simplify well loading
15% Ficoll (type: Pharmacia)		3. migrates at predictable rate in water

TAE (Tris-Acetate)

48.4 g	Tris base
11.42 mls	glacial acetic acid
20 mls	0.5 M EDTA pH 8.0

make up to 1 litre

TBE (Tris-borate)

108 g	Tris base
55 g	Boric acid
40 mls	0.5 M EDTA pH 8

make up to 1 litre

(use only for diagnostic gels - not for DNA extraction)

3.8 RNA gel

Note: Formaldehyde vapors are toxic. Use hood.

1. Prepare 1.5% gel

   1.0g molecular biology grade Agarose

   60ml DEPC H_2O

   Heat to 60°C and stir

Add

   7ml 10x MOPS

   2.1ml 37% Formaldehyde (pH >4)

   Mix with stir bar.

   Pour gel. (Solidifies quickly) Set until cloudy (≈20min)

   Use 10 well comb (fits ≈ 50µl on a good day)

2. Prepare samples

Premix:	4.5µl	10x MOPS
	7.9µl	37% Formaldehyde
	22.5µl	Formamide
	34.9µl	
Add:	10.1µl	Max. RNA (0.5 to 10µl)
		(5µl=5µg for Promega RNA Marker)
	45.0µl	(use DEPC water)

   Mix, incubate 56°C for 15 min.

Add:	9µl	Loading Buffer
	9µl	EtBr. (500µg/ml)

   Mix, spin, load into gel.

3. Run gel. 5V/cm x 10 cm i.e.50V. 3h gives good resolution. 2h is OK.

3.9 RNA Markers (Promega)

The following 8 bands are visualised in an ethidium- stained 1% formaldehyde gel / MOPS running buffer with 2µl of the RNA' prepared in formaldehyde / MOPS buffer.

Number of bases: 9488
6225
3911
2800
1898
872
562
363

3.10 Transformation of bacteria (for subcloning)

1. Obtain bacterial strains (XLI Blue for tPA, NM522 for TF) for the plasmids in which DNA inserted (pBSIIkS for tPA, PGEM for TF).
2. Add 40 ml of sterile LB medium to a sterile culture flask . Scrape 1 colony of bacterial cells (above) with a sterilised metal loop and place in LB medium.
3. Put flask in shaking water bath at 37°C. Stop when medium has an O.D. of 0.3 to 0.5 at 600 nm (check small aliquot at 2 hours and then every half hour).
4. Spin cells at 2,500 g for 5 minutes at room temperature.
5. Gently resuspend pellet of cells in 4 mls of 50 µM $CaCl_2$. Bring up to 20 mls with 50 µM $CaCl_2$ (cells now fragile).
6. Incubate for 30 minutes on ice (cells now "competent").
7. Centrifuge cells for 5 minutes at 2,500 g at 4°C. Resuspend in 4 mls ice-cold $CaCl_2$.
8. Store cells overnight at 4°C.
   Store in 30% glycerol if longer term storage (-70°C).if longer term storage required.
9. In a 4 ml snap-top sterile tube, add about 0.1 µg of plasmid DNA to 200 µl of competent cells.
10. Incubate on ice for 30 minutes.
11. Heat shock cells by transferring tube to a 37°C water bath for 5 minutes.
12. Add 1 ml of sterile LB medium and grow at 37°C for 1 hour in shaking incubator.

3.11 Plating transformed cells

1. Use sterilised bent glass rod to spread Xgal (800µg) and IPTG (800µg) on each LB agar plate containing ampicillin ± tetracycline (only for XLI Blue).
2. Add 100-200µL of transformed cells to each plate and spread evenly.
3. Incubate at 37°C overnight.
4. All colonies growing should contain plasmid, as antibiotic resistance is contained in plasmid. White colonies contain the insert in the plasmid, otherwise the blue colour is expressed.
5. Remove single colonies of interest (usually white) next day, with a sterile loop and grow up overnight in ≈15mls of LB medium (shaking, 37°C).

3.12 Transcription Protocol - probe preparation

20 µL of ^{35}S-UTP (10 µCi / µL) Activity greater than 1000 µCi/mmol. (10 µM. UTP) speed vac dry (45 min, medium drying rate) .

(Substitute 1 µL rUTP stock for "cold" RNA synthesis)

Premix 10.5 λ :

4 µL	5x Transcription Buffer
2 µL	100 mM DTT
0.5 µL	RNasin (=RNasin inhibitor, 20 units)
4 µL	rNTP mix (2.5 mM ea) (1:1:1:1 ATP, GTP, CTP, water)

(may also want to try 1λ 100µM rUTP - some protocols use 100µM cold + UTP)

To premix add:

≈1 µL Linearized template (0.2 µg - 1 µg) (gene cleaned)
1 µL Polymerase (15-20 units)
make up to 20 µL / reaction with DEPC water.
Incubate 37-40°C 1 1/2 - 2 hours (Promega suggests for full length transcripts use 22°C)

NOTE: limiting nucleotide should be greater than 12 µM but less than 24 µM (UTP in this case)

3.13 Remove DNA Template

1. Add RQ1 RNase-free DNase. 1 unit/µg template. Use 5 µL. Incubate 15 min 37°C.
2. Add 20 µg tRNA. Use 2 µL Wheat tRNA solution.
3. Bring volume up to 100 µL, with DEPC H_2O.
4. Extract with 1 volume (100 µL) SSC saturated phenol: chloroform: isoamyl alcohol 25:24:1. Vortex. Spin 13K RPM 2 min.
5. Take upper aqueous phase (with RNA) and add to "new" tube. To original tube add chloroform: isoamyl alcohol 1 volume (100 µL) (24:1). Vortex. Spin 13K RPM 2 min. Transfer upper phase to new tube.
6. Remove unincorporated nucleotides and concentrate RNA
   a. Add 1/2 vol (50µL) 7.5M NH4AC (pH should be ≈6)
   b. Add 2.5 vol (250 µL) EtOH. Mix. Store -80°C 30 min.
   c. Spin 13 K RPM 5 min.
   d. Discard supernatant by shaking liquid out.
   e. Resuspend in 100 µL 1M NH4AC . Mix.
   f. Add 2.5 vol (250 µL) EtOH. Mix. Store -80°C 30 min.
   g. Spin 13 K RPM 5 min.
   h. Shake off supernatant.
   i. Wash pellet with 500 µL 70% EtOH. (Store -80°C until ready to use)
   j. Spin 5 min 13K RPM. Discard supernatant being careful not to throw pellet away also.
   k. Air dry 10 min
   l. Resuspend pellet in DEPC water (10 µL). Use or store at -70°C for up to 4 weeks if necessary.
7. Run RNA on denaturing RNA gel to check transcription (≈1µL) and expose overnight on XR plate.
8. Check for incorporation by counting scintillations.

3.14 Slide preparation

Cleaning slides for in-situ hybridisation

1. Place slides in racks.
2. Dip in 1% 1M HCl in 70% EtOH. (For in-situ use DEPC H_2O) 30 min
3. Dip in DEPC H_2O. 30 min
4. Dip in 95% EtOH (240 ml EtOH / 250 ml) 30 min
5. Put in oven 70°C until dry.

Cleaning coverslips (in-situ)

Soak in 10% Nitric Acid Bath 1h. (36ml of 70% HNO_3 / 250ml DEPC H_2O)

Rinse with DEPC H_2O.

Allow to dry.

Autoclave 30 min. Drytime 10 min.

Chrom-Alum Slide preparation

1. Add 0.1g gelatin (Sigma) to 250 ml DEPC H_2O. Dissolve by heating to 60°C .
2. Add 0.1g Chromium K_2SO_4. (ACS). 12 H_2O- turns blue.
3. Dip cleaned slides (see above). Warm solution for 30 seconds.
4. Dry overnight 37°C . Keep covered with foil.
5. Return slides to box. If not dry, will stick.

Poly-L-lysine slide preparation

Dip cleaned slides in 50 µg poly-L-lysine in 10 µM TRIS pH 8 for 30 minutes.

Dry overnight at 37°C.

Store at 4°C.

3.15 In-situ hybridization Protocol - Day 1.

1. Make 4% paraformaldehyde in PBS with millipore water - heat to 65°C in fume hood. Cool and filter. Store at 4°C.
2. Cut sections (20 µm). Mount on Poly-1-lysine coated slides. Air dry 30 min. 20' at RT
3. Fix with paraformaldehyde at 4°C

   Pipet individually onto each slide (1mL/slide). Keep at 4°C to inhibit endogenous ribonucleases.

Prepare Proteinase K mix.

4. Rinse with PBS (300 mL) for 15' at RT

Prepare triethanolamine (used in step 8)

5. Proteinase K digestion (300 mL) 1µg/mL in buffer incubate for 5-20 minutes (see text)

Use 15 mL stock/300 mL Proteinase K buffer

6. Rinse in PBS (300mL) for 15' at RT

7. Dip 2x DEPC H_2O (300mL)

8. Acetylate. Make 0.1 M triethanolamine-HCl (pH8.0) (5.6g/300mL DEPC H_2O)

   Add Acetic Anhydride. 750µL/300mL triethanolamine
   Acetylation blocks positive amino acid groups (reduces electrostatic binding).
   Add slides to step 8 mix. Gently shake. Incubate for 10 minutes at RT.

9. Wash in PBS (300 mL) for 15 minutes at RT

10. Postfix in 4% Paraformaldehyde/PBS (300 mL) for 5' at RT

Prepare prehybridisation mix

11. Dehydrate- 70% EtOH for 4' at RT
    90% EtOH for 4' at RT
    100% EtOH for 4' at RT
    Air dry

12. Prehybridisation. Apply 200 mL/ section. Incubate for 1 hour at. 45°C

Preheat extra prehybridisation mix to 45°C as well.

13. Hybridisation Step.

Make up hybridisation mix by adding 10% dextran by weight to prehybridisation mix. Dissolve dextran. (difficult). Add enough RNA probe to give approximately 5×10^5 counts of probe per 150µL of hybridisation mix.(each section) Heat RNA to 80 to 95°C for 5 minutes to "melt" RNA. Drain prehybridisaton solution from slides, but do not let them dry.

Apply 150µL probe/section .

14. Apply coverslips

15. Incubate overnight at 45°C

3.16 In-situ hybridization Protocol - Day 2.

1.	Preheat TE buffer and RNase buffer at 37°C		
2.	Remove coverslips from incubated slides in 4 x SSC/10 mM DTT.		
3.	Wash in 4 x SSC/10 mM DTT	for 5 minutes	RT
4.	Wash in 4 x SSC10 mM DTT	10 min	RT
5.	Wash in 4 x SSC/10 mM DTT	10 min	RT
6.	RNAse treatment: 20 µg/ml in 0.5M NaCl/1 xTE buffer (keep in special Wheaton dish)	30'	37°C
7.	Rinse 1 x TE / 10 mM β-mercaptoethanol	30'	37°C
8.	Rinse 1 x TE / 10 mM β-mercaptoethanol	30'	37°C
9.	Rinse 2 x SSC / 10 mM β-mercaptoethanol	30'	RT
10.	Rinse 2 x SSC / 10 mM β-mercaptoethanol	30'	RT
11.	Rinse 0.1 x SSC/ 10 mM β-mercaptoethanol	30'	45°C
12.	Dehydrate 70% ethanol / 0.3 M NH_4Ac	4'	RT
	90% ethanol / 0.3 M NH_4Ac	4'	RT
	100% ethanol / 0.3 M NH_4Ac	4'	RT

13. Air dry

Place XR plate on top of slides and store 1-3 days.

Develop film to check for activity and background and coat with photographic emulsion.

3.17 Emulsion Coating of Slides

1. Use a sodium vapor lamp in the dark room which is a minimum of 4 feet away from your working surface.

2. Dilute Ilford K-5 emulsion 1:1 with millipore water and place in a 45°C water bath for 30-45 minutes. The container of emulsion should be covered during the melting period. An alternative is to cover the entire water bath.

3. After melting the mixture, gently stir the emulsion with either a wood stick or a porcelain spatula. The emulsion should be thoroughly mixed with the water.

4. Using the corner of a tissue, carefully go around the edge of the surface of the emulsion to remove any bubbles.

5. Carefully transfer the emulsion to the dipping chamber which should be seated in the 45°C water bath.

6. Dip several blank slides in the emulsion to remove bubbles. Slides should be dipped straight in and out without agitation.

7. Dip experimental slides in the above fashion. After each slide is dipped, wipe the emulsion off the back side of the slide. This is best done with a kimwipe of a paper towel. Use a fresh kimwipe for each slide.

8. Place the dipped slides on a plexiglass slide rack (about 40° angle) and allow them to drain on paper towels.

9. Transfer the slides to black light-tight boxes and allow the emulsion to dry 2-3 hours.

10. Place the slides in black slide boxes which contain 2-3 dessicant capsules (humicaps). Tape the boxes with black electrical tape, wrap the boxes in foil. label. and place in refrigerator (4°C), no light bulb, for incubation period.

3.18 Development of Emulsion Dipped Slides

1. Bring the slides to room temperature prior to development. (Take the black boxes from the refrigerator and leave at room temperature sealed and with the proper safe light on)

2. Use all developing, rinsing and fixing solutions at 15°C.

3. Develop as follows:

D-19 undiluted for 2.5 min

water wash x 1 min

30% Na Thiosulfate (1) x 10 min.

30% Na Thiosulfate (2) x 10 min.

running water for 60 min.

If the slides are not cleared at this point, i.e. there is a residue of gelatin from the emulsion, dehydrate and rehydrate slides prior to counterstaining.

Suggested counterstain is freshly filtered Richardson Stain for 3-4 min, several water rinses until clear,

dehydrate and coverslip in permount.

3.19 In-situ solutions

Proteinase K buffer

20 mls 1M Tris HCl (pH 7.6)

4 mls 0.5 M EDTA

make up to 300 mls with DEPC water

Prehybridisation solution (to make 1 ml)

2.5M NaCl	120λ
3M NaAc (pH 5.2)	6.7λ
0.5M EDTA (pH 8)	10λ
Formamide	0.5ml
50x Denhardt's	20λ
1M DTT	40λ
20 mg/ml tRNA	12.5λ
10 mg/ml DNA	25λ
20% SDS	5λ

make up 1 ml with DEPC water

Hybridisation solution

Prehybridisation solution + 10% Dextran dissolved at 45°C.

Probe added to give ≈ 10^6 cpm/section i.e. 10^6 cpm/150 µl

Appendix 4 IMMUNOHISTOCHEMICAL EXPERIMENTS

Appendix 4.1

Focal ischaemia/reperfusion experiments

Plasmalyte perfusion for immunohistochemistry

	174	213	205	218	244
Date of procedure	1/9/93	4/8/93	20/10/93	27/7/93	26/1/94
Duration of ischaemia/reperfusion	0 (control)	3/24	3/1	3/1	3/1
Neurological score					
Baseline	100	100	100	100	100
1 Hour	-	27	27	42	42
2 Hour	-	27	27	27	42
3 Hour	-	27	27	27	42
Reperfusion	-	81 (24 hours)	27	27	42
Full blood count					
Baseline					
Haemoglobin (gm/dL)	12.5	12.7	13.7	12.3	12.5
Platelets (per µL)	242,000	186,000	336,000	345,000	435,000
WBC (per µL)	4,800	13,000	9,100	8,600	11,100
1 Hour WBC	-	16,000	8,600	28,400	20,300
3 Hour WBC	-	27,000	9,500	33,600	26,000

Appendix 4.2

Focal ischaemia /reperfusion experiments

Paraformaldehyde fixation for in-situ hybridisation

	245	233	242
Date of procedure	24/2/94	15/3/94	24/3/94
Duration of ischaemia/reperfusion	3:1	3:1	3:24
Neurological score			
Baseline	100	100	100
1 Hour	47	27	77
2 Hours	47	27	77
3 Hours	47	27	77
Reperfusion	47	27	77
Full blood count			
Baseline			
Haemoglobin (gm/dL)	10.7	12.3	12.2
Platelets (per μL)	605,000	321,000	174,000
WBC (per μL)	14,700	7,400	15,700
1 Hour WBC	30,600	35,400	39,400
3 Hour WBC	24,100	21,500	37,000

Appendix 4.3

Focal ischaemia experiments

Deficit sustained at time of device implantation

(Plasmalyte perfusion)

	246	239	240
Date of procedure	15/12/93	13/1/94	12/1/94
Duration of ischaemia	7 days CVA	28 hours CVA	7 days CVA
Neurological score			
Baseline	76	27	81
Full blood count			
Baseline			
Haemoglobin (gm/dL)	12.6	14.7	12.6
Platelets (per μL)	704,000	331,000	402,000
WBC (per μL)	7,200	8,200	7,000

Appendix 4.4

Microvascular patency study

(India Ink)

	#235
Date of procedure	20/12/93
Duration of ischaemia/reperfusion	1:1
Neurological score	
Baseline	100
1 Hour	46
Full blood count	
Baseline	
Haemoglobin (gm/dL)	12.5
Platelets (per μL)	459,000
WBC (per μL)	22,500
1 Hour WBC	30,600

Appendix 5 DNA SEQUENCES

Appendix 5.1

Sequence of sub-clone of Tissue Factor pJH24

Using M13 promotor (T7)

GTCGGGTTG-TAATACGACTCACTATAGGGGGAATTGGGCCCGACGTCGC

↑T7

ATGCTCCTCTAGACTCGAGGAATTCCCCTTTCTCCTGGCCCATACACTCTA

↑bp780 of tPA

CCGGGCTGTCTGTACTCTTCCGGTTAACTGTTCGGGAGGGAATCACTGCTT

GAACACTGAAACAGTAGTTTTCTCCTTTATCCACATCAATCAAAAACTCAT

TAGTGTTTGTTTTGGCTGTTTTCTTTCCTGAACTTGAAGATTTCCAATAATA

AAGTGTATAAATTAAGTCCTTGCCAAAAACATCCCGGAGGCTTAGGAAAG

TGTTGTTCCTTCTGACTAAAGTCCGTTCATCTTCTACG

Appendix 5.2

Sequence of sub-clone of Tissue Factor pJH24

Using M13r promotor (SP6)

CATGATTACGCCAAGCT-ATTTAGGTGACACTATAGAATACTCAAGCTATG

↑SP6

CATCCAACGCGTTGGGAGCTCTCCGGATC-CAACTGGTAGACATGGAGAC

↑bp22 of tPA

CCCTGCCTGGCCCCGGGTCCCGCGCCCCGAGACCGCCGTCGCTCGGACGC

TCCTGCTCGGCTGGGTCTTCGCCCAGGTGGCCGGCGCTTCAGGNACTACA

AATACTGTGGCAGCATATAATTTAACTTGGAAATCAACTAATTTCAAGAC

AATTTTGGAGTGGGAACCCAAACCCGTCAATCAAGTCTACACTGTCAAAT

AAGCACTAAGTCAGGAGATTGGAAAAGCAAATGCTTTTT

Appendix 5.3

Sequence of sub-clone of tPA

Using M13 promotor (T7)

CGTNATACGACTCACTATAGGGCGAATTGGAGCTCCACCGNGGTGGCGGT

T7 site on pBS IIKS

CGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGG-AATTCCATGATCCTGA

↑bp804 of tPA

TAGGCAAGGTTTACACAGCACAGAACCCCAGTGCCCAGGCACTGGGCCTG

GGTAAACATNATTACTGNCGGAATCCTGATGGGGATGCCAAGCCCTGNTG

TCACGTNCTGATGAACCGCAGGCTGACGTGGGAGTACTNTGATGTGNCCT

CCTGCTCCACCTGNGGNTCTGAGACAGTACAGACAGCCTCAG

Appendix 5.4

Sequence of sub-clone of tPA

Using M13r promotor (T3)

GGGAACAAAAGCTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGAT

T3 site on pBSII KS

AAGCTTGATATCG-AATTCCTTAATGGACAATGTATTTTTCGACTTCAAAT

↑bp1279 of tPA

TTCTGCTCCTCCTCGCCAGGGACCACCCGGTATGTTCTGCCCAAGATCACC

GTCAGGTGGTGGGGCGGAAACCTCTCCTGGAAGCAGTGGGCGGCAGAGA

GAATCCAGCAGGAGCTGATGAGTATGCCCCCGCACAGGAACCGCTCTCCG

GGCGACCTCCTGTGCTTGGCAAAGATGGCAGCCTGCCAGGGGTGGGAGGC

GATGTCGGC

Appendix 6 PUBLICATIONS

Publications

Okada Y, Copeland BR, Fitridge RA, Koziol JA, & del Zoppo GJ.
Fibrin contributes to microvascular obstructions and parnchymal changes during early focal cerebral ischaemia and reperfusion.
Stroke 1994; 25: 1847-1854.

Published abstracts

Hamann G, Okada Y, Fitridge RA, del Zoppo GJ.
Microvascular basal lamina disappears during cerebral ischaemia and reperfusion.
Accepted for presentation at the American Heart Association meeting 1995; received Robert G Siekert Award of the American Heart Association 1994. Manuscript in preparation.

Publications prepared during period of research fellowship

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