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Gene activation and protein expression following ischaemic stroke: strategies towards neuroprotection

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Abstract

Current understanding of the patho-physiological events that follow acute ischaemic stroke suggests that treatment regimens could be improved by manipulation of gene transcription and protein activation, especially in the penumbra region adjacent to the infarct. An immediate reduction in excitotoxicity in response to hypoxia, as well as the subsequent inflammatory response, and beneficial control of reperfusion *via* collateral revascularization near the ischaemic border, together with greater control over apoptotic cell death, could improve neuronal survival and ultimately patient recovery. Highly significant differences in gene activation between animal models for stroke by middle cerebral artery occlusion, and stroke in patients, may explain why current treatment strategies based on animal models of stroke often fail. We have highlighted the complexities of cellular regulation and demonstrated a requirement for detailed studies examining cell specific protective mechanisms after stroke in humans.

Keywords: stroke - gene expression - angiogenesis

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Introduction

Ischaemic stroke is a leading cause of death and disability worldwide. In more than 80% of cases, it results from a transient or permanent reduction in cerebral blood flow, caused by occlusion of a cerebral artery by an embolus or local thrombosis [1]. Two-thirds of patients survive the initial event but are left with significant degrees of sensorimotor, cognitive, or other impairment. The ischaemic penumbra is a region of tissue surrounding the ischaemic core, that has been identified for upwards of 48h after stroke in patients, and that has intermediate perfusion, where cells depolarize intermittently [2-4]. Without treatment, the penumbra often progresses to infarction owing to the effects of ongoing excitotoxicity, spreading depolarization and post-ischaemic inflammation. Maintenance of perfusion pressure in this region and hence survival of neurones within this dynamic area of tissue is critical for the minimisation of long-term damage. In this review, we have examined current features of stroke development based on animal models or *in vitro* experiments, as well as the limited work demonstrating changes observed after stroke in humans.

Excitotoxic and inflammatory responses

Within minutes of arterial occlusion, the affected area of brain tissue becomes hypoxic and hypoglycaemic. Rapid release of glutamate from presynaptic nerve terminals and astrocytes causes overstimulation of N-methyl-D-aspartate (NMDA) and glutamate receptors [5,6]. This excitotoxicity results in influx of Ca^{2+} and Na^{+} followed passively by movement of Cl^{-} and water, culminating in oedema, plasma membrane failure and neuronal necrosis (Fig. 1). Increase in Ca^{2+} mediates activation of phospholipase C/A_2, cyclooxygenase-2 (COX-2), and lipolysis followed by signal transduction intermediates (*e.g.* mitogen activated protein kinase; MAP kinase), nitric oxide, and lipid peroxidation products, respectively, resulting in tissue damage and neuronal necrosis. Oxygen free-radicals, Ca^{2+} and inducible nitric oxide synthase (iNOS), as well as other hypoxia-induced molecules, also serve as signalling molecules that trigger the inflammatory process, which occurs within hours of the initial insult [7]. Rapid Induction of transcription factors occurs in damaged astroglia, microglia, endothelial cells (EC), leukocytes and peripherally derived immune cells resulting in increased expression of inflammatory cytokines and chemokines (Fig. 2). Nuclear factor κB (NF- κB), a major protagonist, activates tumour necrosis factor α (TNF- α) and interleukins 1α , 1β , and 6 (IL-1α , IL-1β , IL-6) [8]; hypoxia inducible factor 1 (HIF-1) induces vascular endothelial cell derived growth factor (VEGF), enhancing blood-brain barrier leakage and oedema [9]; interferon regulatory factor 1 (IRF-1) stimulates production of gamma interferon (γ -interferon) which stimulates macrophages [10], whilst activation of either signal transducers and activators of transcription (STAT)-1 or 3 results in overproduction of platelet-activating factor (PAF), monocyte chemoattractant protein-1 (MCP-1) and intercellular adhesion molecule (ICAM-1) [11]. Strong phospho-STAT-1 staining of TUNEL-positive (apoptotic) neurones has been shown in peri-infarcted areas up to 24h following middle cerebral artery occlusion (MCAO) in rats [12] (Fig. 3). Furthermore, STAT-1 knockout mice demonstrated a significantly smaller infarct volume, suggesting a role in cell death [13]. Prostaglandin E2 produced *via* cyclooxygenase and lipolysis can also induce inflammation by up-regulation of TNF-α and IL-6 [14].

Up-regulation of inflammatory cytokines induces expression of adhesion molecules including intracellular adhesion molecule-1 (ICAM-1), platelet EC adhesion molecule (PECAM-1) and EC leukocyte adhesion molecule (ELAM-1) on the EC surface, resulting in neutrophil binding and migration to the brain parenchyma [15]. Macrophages and monocytes follow neutrophils into the ischaemic brain, aided by chemokines such as IL-8 and monocyte chemoattractant protein 1 (MCP-1) produced by damaged brain cells. Within 24h after the infarct, large numbers of inflammatory cells are found predominantly around the infarct, and in particular in the penumbra where they may contribute to brain injury by microvascular obstruction [16], and by producing neurotoxic mediators which include reactive oxygen species (ROS) and nitric oxide (NO) [17, 18].

One study, however, demonstrated that infiltrating leukocytes did not appear to contribute to the infarct size after MCAO in a rat model of stroke [19].

In view of the absence of investigations into the above parameters in human patients, it is not possible to evaluate their full significance in stroke in man. Therapies aimed at reducing excitotoxicity for example by attenuation of the NMDA receptor, have shown promise, but must be delivered within 1-2h after stroke in rodent models [20]. Pharmacological interventions aimed at reducing excitotoxicity and inflammation after stroke, that were entered into phase III clinical trials have been unsuccessful [21]. Inhibitors of NMDA receptors, ion channels and glycine were ineffective probably because blockade of normal synaptic transmission was detrimental to neuronal survival [22]. Strategies employed to reduce the inflammatory response have a longer time-window over which they can be effective. However the secondary, beneficial effects in terms of tissue repair and remodelling would be lost. For example, macrophages actively remove dead cells, whilst a reduction in growth factor release in the vicinity of the infarct and also expression of CD34 positive EC progenitor cells might impair revascularisation [23, 24]. Recent reviews have focussed on the use of inflammatory markers as predictors of brain damage and recovery. For example, plasma IL-6 levels predict neurological deterioration and infarct volume, whilst matrix metalloproteinase-9 (MMP-9) levels are associated with the efficacy of thrombolytic therapy [25]. Detailed examination of changes in expression of these markers is hampered owing to the ethical difficulties in obtaining tissue samples from patients immediately following death from acute stroke.

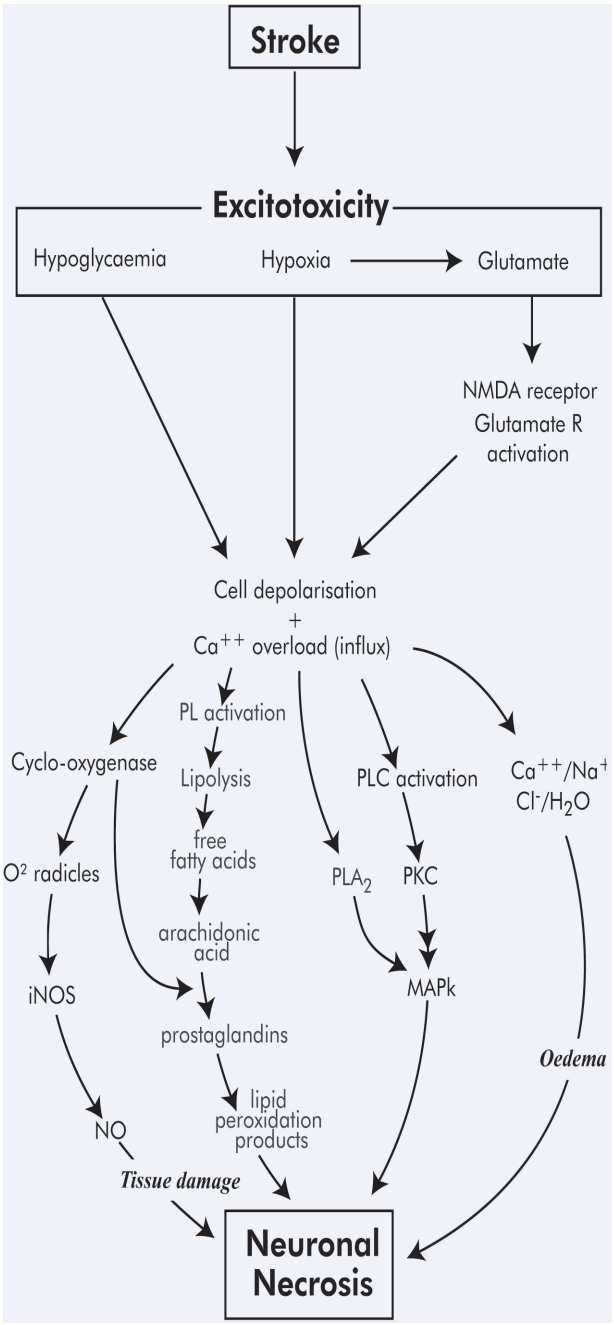

Fig. 1 Mechanisms through which excitotoxicity results in neuronal necrosis following acute ischaemic stroke. Excitotoxicity results in cell depolarization, increased Ca^{2+} influx and subsequent activation of intracellular signalling pathways. Excessive production of cyclo-oxygenase results in release of nitric oxide, whilst phospholipases activate MAP kinase, and simultaneously increase expression of lipid peroxidation products, culminating in neuronal necrosis. Abbreviation: iNos, inducible nitric oxide synthase; NO, nitric oxide; PKC, protein kinase C; MAPK mitogen activated protein kinase; PLC, phospholipase C; ROS, reactive oxygen species; NMDA, N-methyl-D-aspartate.

Induction of neuronal apoptosis

Many susceptible neurones, particularly in the penumbra region, undergo apoptosis, although the mechanisms of this process are not fully understood [26, 27]. Briefly, excitotoxicity, and in particular, excessive production of oxygen free-radicals, results in mitochondrial permeability transition (MPT) leading to disruption of the mitochondrial inner membrane and activation of transcrip-

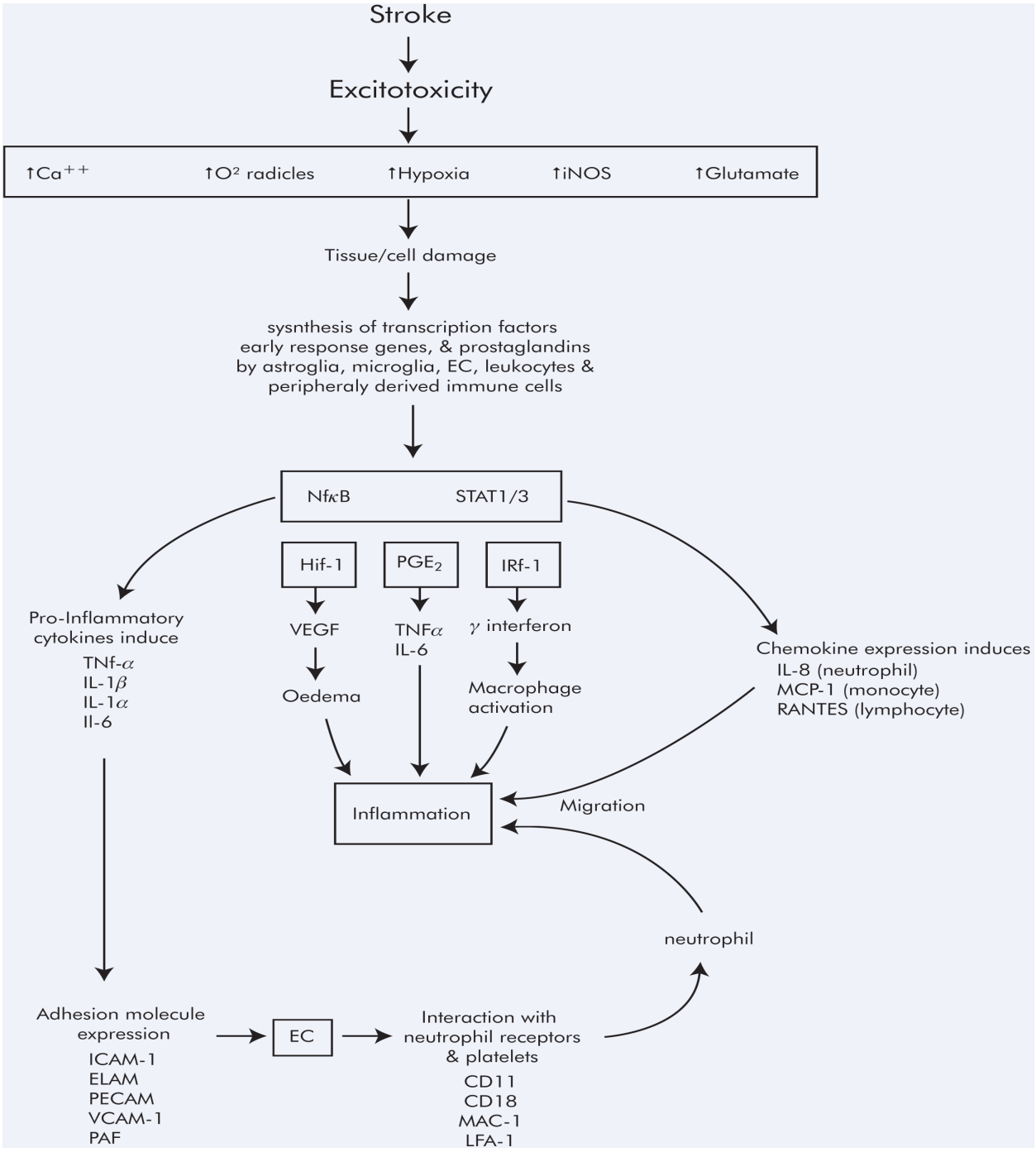

Fig. 2 Inflammatory pathways associated with acute ischaemic stroke. Tissue damage caused in part by the effects of excitotoxicity, induces expression of nuclear transcription factors such as NF- κB in a variety of parenchymal and immune cells. Subsequent synthesis of pro-inflammatory cytokines and chemokines, in association with EC adhesion molecule expression, results in migration of polymorphonuclear leukocytes and lymphocytes to the infarcted area resulting in inflammation. Abbreviations: NF-κB, nuclear factor kappa-B; STAT, signal transducers and activators of transcription; HIF, hypoxia inducible factor; PGE2, prostaglandin E2; IRF-1, insulin responsive factor-1; TNF-α, tumour necrosis factor-alpha; IL, interleukin; ICAM-1, intracellular adhesion molecule; ELAM-1, endothelial leukocyte adhesion molecule; PECAM-1, platelet endothelial cell adhesion molecule; VCAM, vascular cell adhesion molecule; PAF, platelet activating factor; MCP-1, monocyte chemoattractant protein; RANTES, regulated upon activation normal T-cell expressed and secreted; iNOS, inducible nitric oxide synthase; ROS, reactive oxygen species.

tion factors including NF- κB and activating transcription factor-2 (ATF-2). This induces post-translational modification and translocation to the outer mitochondrial membrane, of members of the pro-apoptotic Bcl-2 family, including Bax, Bcl-2 antagonist of cell death (Bad) and Bcl-2 homology domain 3 (BH3)-interfering domain death agonist (Bid), which form channels allowing the release of cytochrome c from the mitochondrial intermembrane space. Release of cytochrome c is the main trigger for mitochondrial associated apoptosis [28-30] (Fig. 4). Cytochrome c induces oligomerization of apoptosis activating factor-1 (APAF-1), subsequent binding with pro-caspase-9, activation of caspase-9 and finally binding and activation of capase-3, which cleaves poly (ADP-ribose) polymerase (PARP) and inhibitor of caspase-activated deoxyribonuclease (ICAD), amongst others, and initiates apoptosis [28, 31].

Other components of the excitotoxic-inflammatory cascade can also contribute to apoptosis. For example, activation of p53 by HIF-1 after hypoxia, and of cysteine proteases (*e.g.* calpain) following increased Ca^{2+} influx, leads to further up-regulation of Bax and subsequent release of mitochondrial cytochrome c [32, 33]. Increased expression of pro-caspases-1, 2, 3, 6 and 8 as well as cleaved caspase-3 occurred 12 and 24h after MCAO in rat penumbral neurones undergoing apoptosis [29, 31]. Cleaved caspase-3 expression and associated neuronal apoptosis were notably reduced in the penumbra region in the presence of the nucleoside, citicoline (CDP-choline; Fig. 5).

Release of pro-inflammatory cytokines including TNF- α and IL-1 activate a number of intracellular signalling pathways, which result in neuronal apoptosis. TNF- α activates TNF-receptor-associated death domain (TRADD), whilst Fas ligand, secreted by the action of matrix metalloproteinases, associates with Fas-associated death domain (FADD) and subsequently the death domains of these proteins interact with the death effector domains of procaspase-8, cleaving it and in turn activating downstream effector caspases and apoptosis [34, 35]. The process may be partly mitochondrial dependent, since caspase-8 can cleave Bid, resulting in release of mitochondrial cytochrome c and activation of pro-caspase-9 followed by pro-caspase-3. Similarly, cytokine activation of MAP kinase pathways operating through

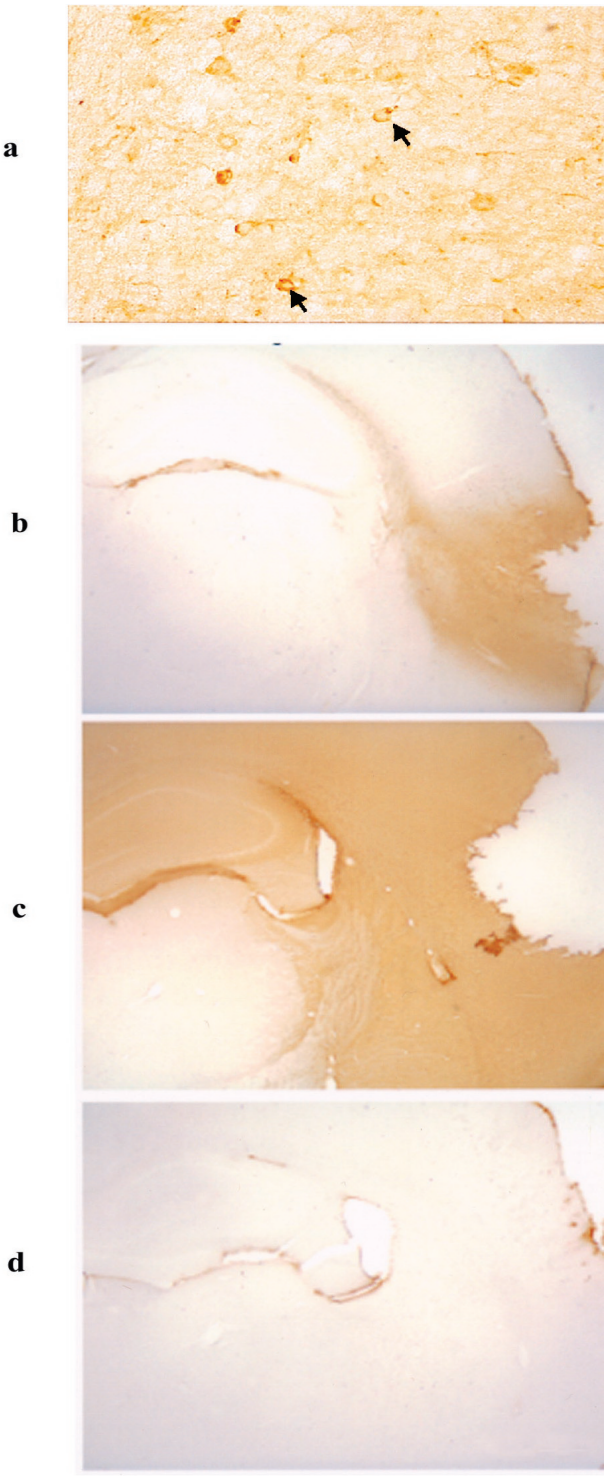

Fig. 3 Expression of phosphorylated STAT-1 after middle cerebral artery occlusion in a rat. (a) Strong staining of neurons (arrow x 100) and in some glial cells 12h after infarct. (b-d), gross morphological appearance of phosphorylated STAT-1 staining, 12h, 24h, and 1 month after infarct. Staining was limited to neurons in the old peri-infarcted area after 1 month (for further details see reference [12]).

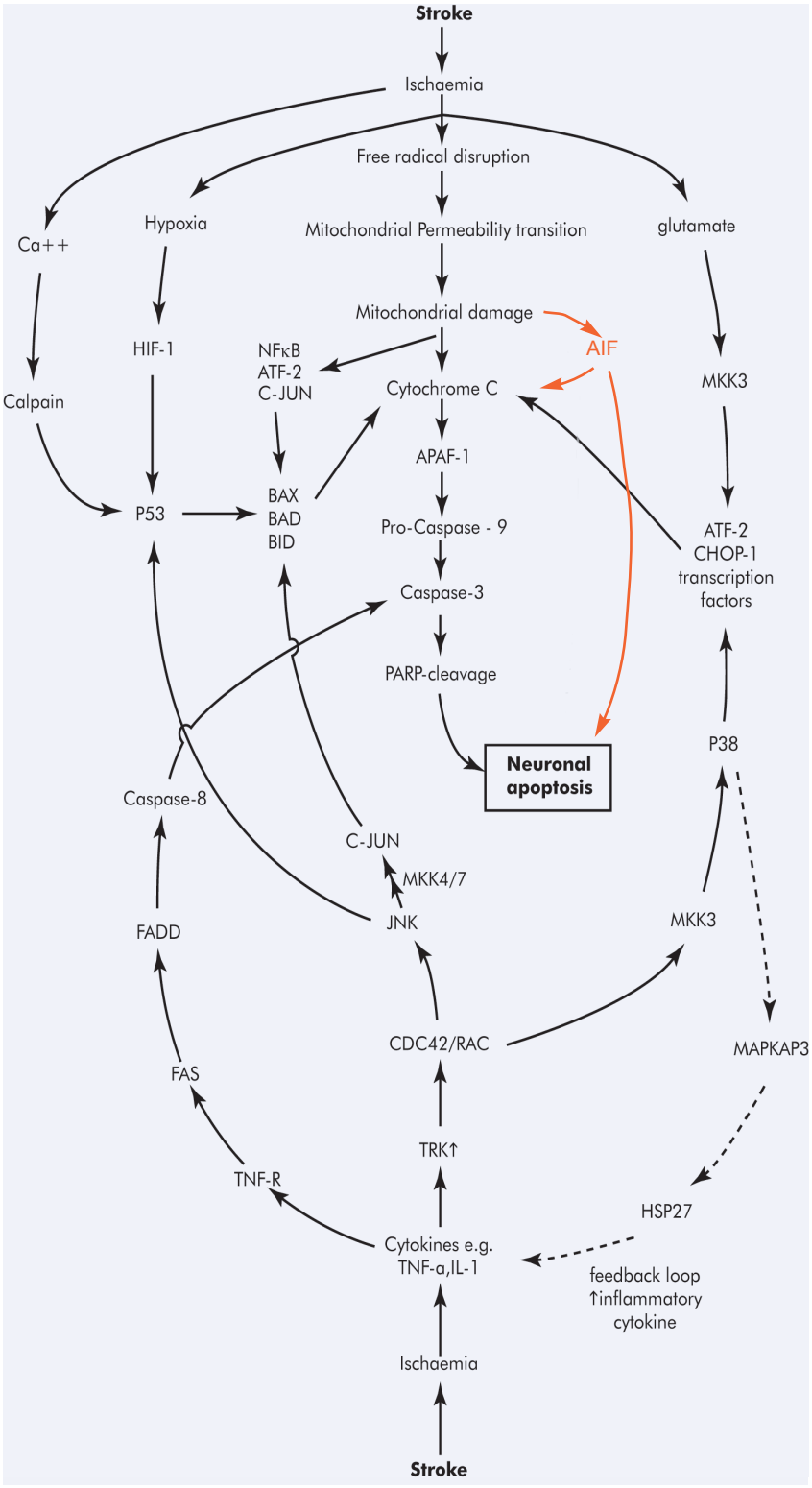

Fig. 4 : Pathways of neuronal apoptosis activated after acute ischaemic stroke. Ischaemia induces mitochondrial permeability transition following free radical disruption, resulting in mitochondrial damage and subsequent release of cytochrome c. This activates the caspase pathway culminating in cleavage of substrates including poly (ADP-ribose) polymerase and apoptosis. Hypoxia, Ca^{2+} influx and increased glutamate as well as over-production of cytokines can promote the expression of a variety of transcription factors and signalling intermediates which increase mitochondrial release of cytochrome c *via* pro-apoptotic members of the Bcl-2 family. A novel pathway involving mitochondrial over-expression of AIF leading to non-caspase mediated neuronal apoptosis has recently been identified. Red arrows signify non-capase mediated apoptosis. Abbreviations: AIF, apoptosis-inducing factor; HIF-1, hypoxia inducible factor-1; NF κB, nuclear factor kappa-B; APAF-1, apoptosis activating factor-1; MKK3, map kinase kinase-3; ATF-2, activating transcription factor-2; CHOP-1, growth arrest and DNA damage-inducible gene 153, JNK, c-jun N terminal kinase; Trk, tropomyosin-related kinase, TNF-α, tumour necrosis factor-alpha; IL, interleukin; FADD, fas-associated death domain protein; HSP, heat shock protein; MAPKAP kinase, map kinase-activated protein kinase; TRADD, TNF-receptor-associated death domain.

c-jun N-terminal kinase (JNK) and p38 MAP kinase, is strongly associated with apoptosis [36]. A chronic increase in phosphorylation of both JNK and p38 in reactive neurones in the penumbra region after MCAO in the rat has been observed [12]. The precise upstream regulators/receptors of JNK activation after ischaemia have not been described. However, its substrate, c-jun, as well as its downstream regulation of p53 have been associated with increased neuronal apoptosis *in vitro*

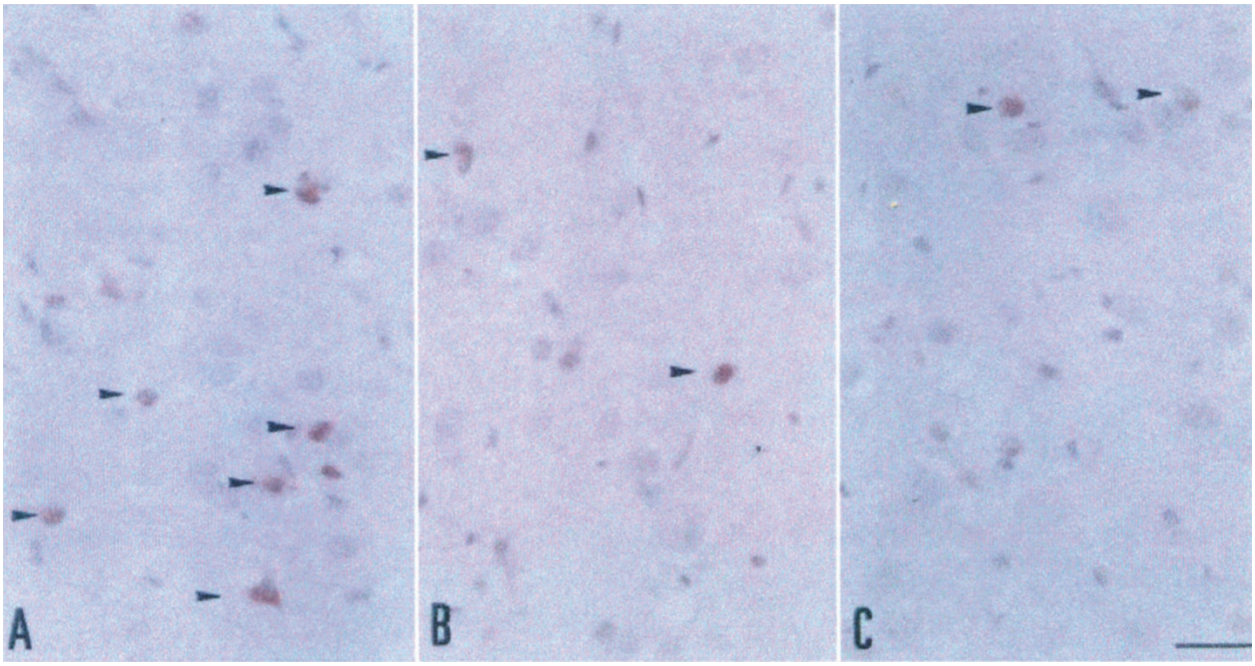

Fig. 5 Expression of cleaved caspase-3 in penumbra tissue after rat middle cerebral artery occlusion (MCAO). (a) Penumbra tissue stained with anti-caspase-3, 12h after infarct. (b) Similar tissue from a rat treated with citicoline prior to MCAO, and (c) immediately after MCAO. A notable reduction in caspase positive neurons was seen in citicoline treated rats (arrows; x 100) (see reference [31]).

[37-39]. Inflammatory cytokines, as well as glutamate, can activate neuronal p38, stimulating transcription factors, ATF-2 and growth arrest and DNA damage-inducible gene 153, (CHOP-1) and mediating cell death *via* increased cytochrome c expression [40]. Furthermore, p38 mediated activation of MAP kinase activated protein kinase 3 (MAPKAP3) stimulates heat shock protein 27 (HSP-27) and as a consequence can up-regulate production of inflammatory cytokines [41]. Further studies are needed to elucidate the mechanisms that control this pathway and to establish the role of p38 in neuronal death.

Non-caspase mediated induction of apoptosis after stroke, which involves mitochondrial release of apoptosis-inducing factor (AIF) in ischaemic conditions, and subsequent DNA fragmentation, has also been described, although the exact mechanisms have yet to be defined [42, 43]. Deregulation of cyclin dependent kinases (CDKs) can induce apoptosis in neurones by modulation of cell cycle progression, however, CDK5, which is not involved in cell cycle control, has recently been shown to promote neuronal PC12 cell death *via* activation of p53 [44, 45]. This pathway might represent a novel mechanism involved in regulation of stroke-induced neuronal apoptosis.

Neuroprotective mechanisms

The brain also activates neuroprotective mechanisms in an attempt to counteract the damaging effects of excitotoxicity and inflammation. A number of neurotrophic factors are up regulated after ischaemic stroke, and may be synthesised and released by neurones, infiltrating leukocytes and microglia. *In vitro* studies using rat embryonic hippocampal neurones have shown that nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF), activate a signal transduction pathway involving phosphor-inositol 3-kinase (PI-3K) and Akt, which is an indirect inhibitor of pro-apoptotic p53 and Bad. NGF and BDNF also stimulate phospholipase C (PLC)-protein kinse C (PKC) pathways, which activate survival pathways involving NF-κB and anti-apoptotic members of the Bcl-2 family [46-48]. Basic fibroblast growth factor (FGF-2) and VEGF acti-

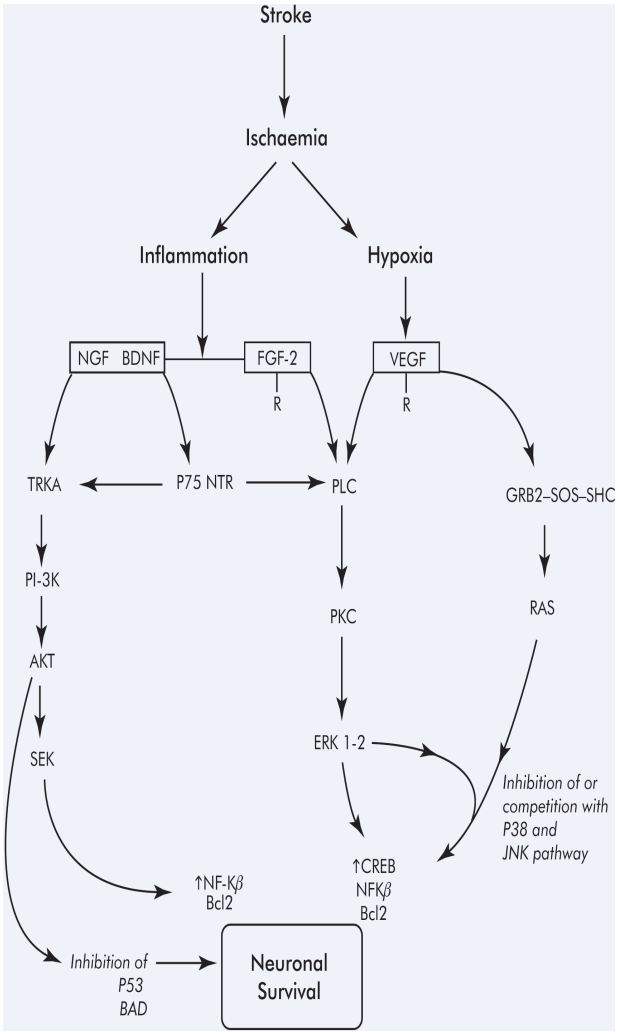

Fig. 6 Potential mechanisms of neuroprotection after acute ischaemic stroke. Inflammation results in increased expression of neurotrophic factors including NGF and BDNF, which bind to the TrkA and neurotrophin receptors respectively and activate a signalling pathway through PI-3K and Akt inhibiting the action of pro-apoptotic proteins p53 and Bad, and inducing pro-survival factors NF-κB and Bcl-2 expression. Growth factors produced during inflammation and hypoxia, together with BDNF activate conventional MAP kinase pathways, which oppose the effects of pro-apoptotic JNK and p38 signalling, and also stimulate further expression of survival factors such as CREB, NF κB and Bcl-2. Abbreviations: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; TrkA, tyrosine kinase A; PI-3K, phospho- inositol-3 kinase; BAD, Bcl-2 antagonist of cell death; NF-κB, nuclear factor-kappa B; PLC, phospholipase C, NTR, neurotrophin; FGF-2, fibroblast growth factor-2; VEGF, vascular endothelial cell growth factor; Grb2, growth factor receptor-bound protein-2; SOS, son of sevenless; JNK, c-jun N-terminal kinase; CREB, cyclic AMP binding protein; ERK1/2, early response kinase 1/2; PKC, protein kinase C.

vate the MAP kinase pathway (ERK1/2) through PLC or ras, stimulating production of anti-apoptotic proteins, Bcl-2, cyclic AMP binding protein (CREB) and NF- κB [49, 50] (Fig. 6). Indirect evidence suggests that increased expression of ras and ERK1/2 might counteract the apoptotic effects of both p38 and JNK [51]. A strong association of phosphorylated MAP kinase (ERK1/2) with neurones in the penumbra region has been demonstrated after stroke [12, 52]. However, the activation was transient (<24h) and a direct association with cell survival was not demonstrated. The mechanisms of these responses require further investigation.

In the above studies, different models of stroke have been employed, including the *in vitro* culture of primary cells and animal models. Interestingly, multiple and sometimes opposite functions have been attributed to individual proteins. For example, inflammatory cytokines such as TNF, contribute to an extension of infarct size and neuronal apoptosis *in vivo* [53], but also exhibit neuroprotection against calcium influx mediated through NMDA receptors *in vitro* [8] and through activation of the EGF receptor in a rat model [54]. Other studies in a mouse model of MCAO showed that anti-apoptotic protection through Bcl-2 resulted in a build up of pro-caspase-9 and promoted cell death, suggesting that simple therapeutic inhibition of individual signalling intermediates may not be sufficient to provide neuroprotection [55]. Similarly, glutamate induced excitotoxicity and neuronal damage *in vitro*, were reduced by inhibitors of MAP kinase, which is surprising, since growth factor stimulation of MAP kinase is neuro-protective [56]. Growth factor withdrawal is also associated with activation of apoptotic pathways, suggesting that modulation of either levels and mixtures of cytokines, the time of expression, or the signal transduction pathways they initiate could be sufficient to affect neuronal survival [57].

Since there have been very few studies describing changes in expression of pro- and anti-apoptotic molecules following ischaemic stroke in man, therapeutic neuroprotection has so far been based on results from animal models of stroke, such as those described above. These treatments have had very limited success in human trials [58, 59]. Success of these agents is time-dependent,

with trials suggesting treatment times in man as being too late if following predictions from animal models. The potential benefits of neuroprotective therapeutic intervention have recently been highlighted by the positive results seen using novel protein transduction technology in preclinical studies [60]. Protein transfer domains (derived from HIV-1 Tat), attached to Bcl-xl, were effectively delivered across the blood-brain barrier and significantly reduced infarct size and caspase activation in a mouse model of stroke [61]. A systematic study of neuroprotective and apoptotic protein de-regulation in patients after stroke is still lacking. However, chronically increased phospho-ERK1/2 expression has been reported in surviving cortical neurons in the penumbra region of patients following ischaemic stroke [62] (Fig. 7).

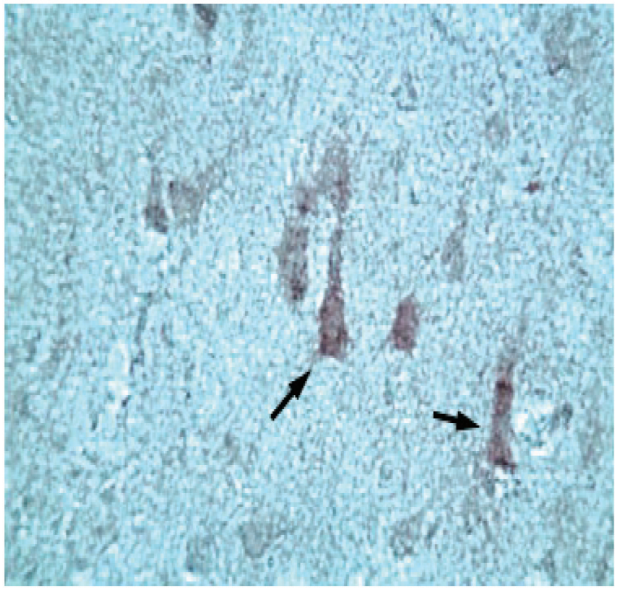

Fig. 7 Intensive staining of phospho-ERK1/2 in neurons arrows of grey matter penumbra in tissue from a patient 3 days after acute ischaemic stroke (see reference [62]).

Revascularisation and tissue reperfusion after stroke

Reperfusion and collateral revascularization of potentially viable tissue could be an important factor in determining patient recovery and therefore thrombolysis may form part of a useful treatment regime. Krupinski *et al* [63-65] demonstrated increased angiogenesis that was associated with tissue survival, in the penumbra tissue of patients after acute ischaemic stroke (Fig. 8).

The revascularization process after MCAO has been described in a rat model using brain vascular casts [66]. The data suggested that new blood vessels initiated through vascular buds, formed regular connections with intact microvessels within one week of ischaemia, the patterns being similar to those seen in the normal brain (Fig. 9). This group and others showed that apoptosis within damaged EC might be necessary to regulate the process [66, 67]. It has been reported that arteriolar collateral growth and new capillaries supported restored perfusion in the ischaemic border after ministroke [68], and in the cortical region after photothrombotic ring stroke in rats [69].

Significant quantities of angiogenic growth factors are secreted by inflammation associated infiltrating macrophages, leukocytes and damaged blood platelets, which probably helps to maintain increased circulatory expression after stroke [70, 71]. Specific up-regulation of angiogenic factors (*e.g.* VEGF, FGF-2), occurs in EC, in response to hypoxia associated activation of second messenger pathways involving ERK1/2, p38 and JNK MAP kinases [72, 73], (Fig. 10). Cytokines, including TNF- α and IL-1, released following stress and inflammation, induce transcription of growth factor mRNA through the same signalling intermediates [74]. These cytokines also stimulate increased expression of angiopoietin-1. Angiopoietin-1, which binds to the EC-specific receptor, tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 2 (Tie2), can mediate cell survival through PI-3K and the serine-threonine kinase Akt (or Protein Kinase B), and cell migration *via* growth factor receptor-bound protein (Grb7)-focal adhesion kinase (FAK), and has been shown to reduce cerebral blood vessel leakage and ischaemic lesion volume after focal cerebral embolic ischaemia in mice [45, 75].

VEGF, perhaps the most potent angiogenic factor, is up regulated within hours of stroke and has a strong influence on the growth of new blood vessels after ischaemia [76, 77]. Unfortunately, its function as a vascular permeability factor also means that localization in the primary ischaemic core causes blood-brain barrier leakage resulting

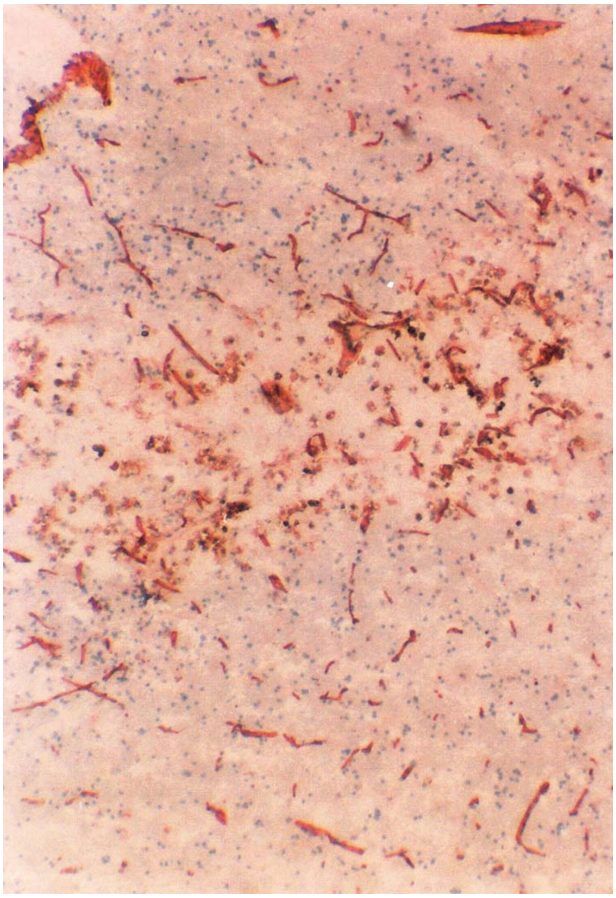

Fig. 8 This figure shows high microvessel density in infracted brain tissue from a patient with acute ischaemic stroke which has been shown to correlate with good prognosis (see references [64, 65]).

in brain oedema [9]. The role of endogenously produced transforming growth factor-β (TGF-β) after stroke remains to be elucidated, however, injection of a selective antagonist (T beta RII-Fc) caused an increase in infarct volume following induction of cerebral focal ischaemia in rats [78]. Injection of TGF-α into rat brains following MCA occlusion resulted in a significant decrease in stroke volume [54]. This effect was ameliorated following pre-injection with the specific epidermal growth factor (EGF) receptor inhibitor 4,5-dianilinophthalimide (DAPH), suggesting that TGF-α was operating through the EGF receptor.

Typical growth factor induced signal transduction pathways bind *via* the Src-homology-2 (SH2) domains of transmembrane tyrosine kinase receptors [79], resulting in activation of signal transduction cascades, which may be complicated by their integration at various levels [80]. EC proliferation, an important feature of angiogenesis, involves activation of PLCγ, Src, PKC and Ras, culminating in the stimulation and subsequent nuclear translocation of MAP kinase (ERK-1/ERK-2) and rapid phosphorylation of early response genes such as Elk-1 [79, 81, 82]. EC can express two forms of the VEGF receptor VEGFR-1 (KDR) and VEGFR-2 (Flt) (reviewed in [83]). However, only cells expressing VEGFR-2 activated MAP kinase, were able to undergo cell proliferation. EC migration also, can be induced through MAP kinases [84], or by a separate transducing mechanism involving membrane bound heterotrimeric G-proteins coupled to phospholipase A2 and arachidonic acid (not shown in Fig. 10). Rho GTPases, activated through multiple types of receptor (*e.g.* G-protein-coupled, tyrosine kinase and cytokine receptors) stimulate cell migration in concert with ras [85]. Activation of p38 and JNK MAP kinases *in vitro* results in stabilization of growth factor mRNA as well as promotion of EC migration. Studies have shown that inhibition of FGF-2 stimulated p38, enhanced neovascularization in the chick chorioallantoic membrane. However, the vessels displayed abnormal features of hyperplasia, suggesting an important role for p38 in the regulation of angiogenesis [86]. Inhibition of FGF-2 induced p38 MAP kinase in mouse spleen EC, prevented tube formation in type I collagen gels, and attenuated both proliferation and migration of those cells [87]. Many of the same angiogenic factors (*e.g.* platelet derived EC growth factor; PDGF, FGF-2 and endothelin-1- ET-1) produced during strokes can also induce proliferation of smooth muscle cells, which have an important role in the revascularization process [88]. Although increased expression of growth factors and cytokines has been shown in a variety of animal models following acute stroke, many of the signalling pathways described above, that are responsible for revascularization have only been subjectively proposed on the basis of *in vitro* culture studies.

Activation of MAP kinase may have a pivotal role in stroke-associated abrogation of apoptosis, controlling angiogenesis and promoting VEGF expression through HIF-1 [72]. It has been shown that a transient (<24h) increase in expression of phosphorylated p38, p-ERK1/2 and JNK MAP

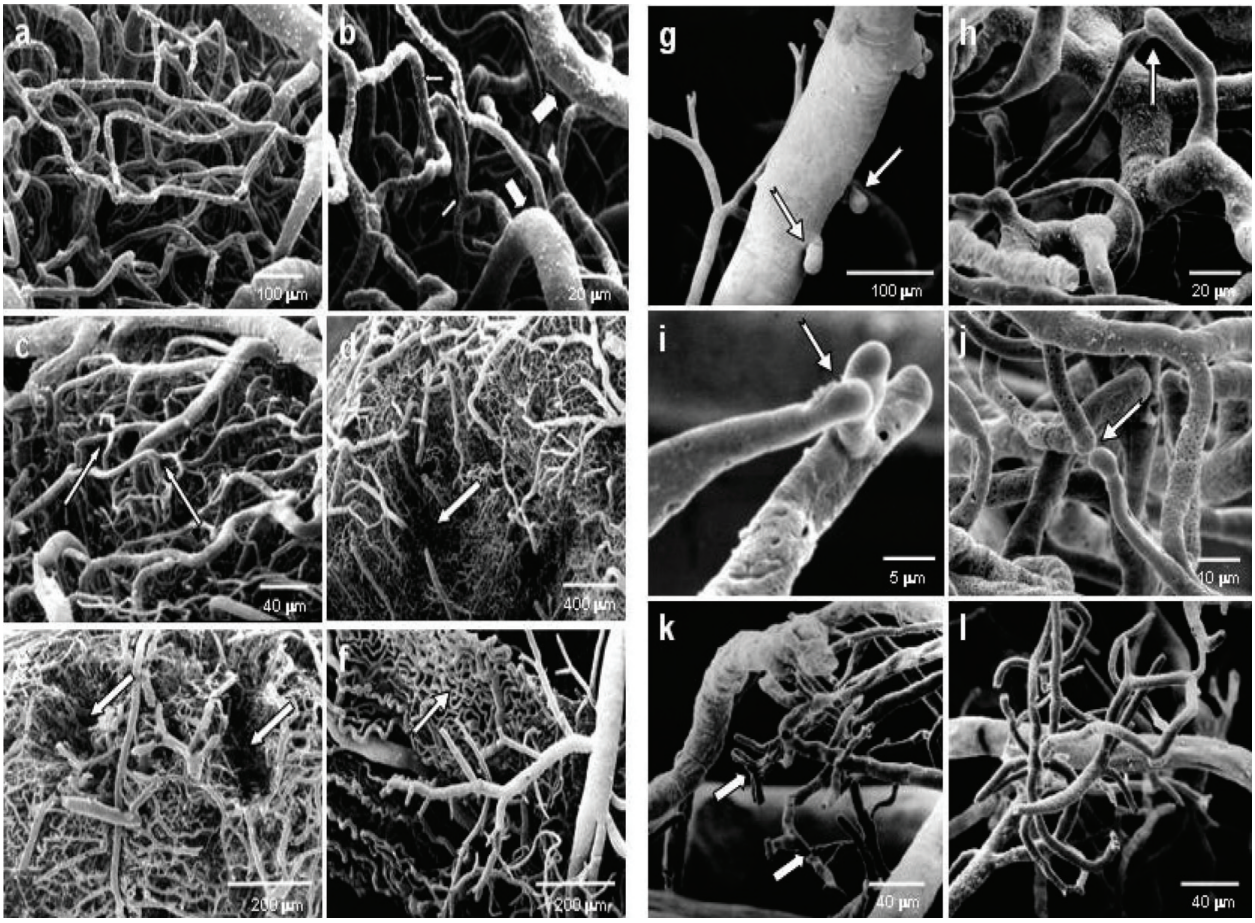

Fig. 9 Scanning electron microscopy of the vascular cast from normal rat brain and following MCAO in a rat model of stroke. (a-c), casts from normal adult rat (size demonstrated by bar) showing leptomeningeal (large arrows) and small penetrating arterioles (small arrows) (a). An extensive micro-vascular network interconnects radially arranged penetrating arterioles and venules (b-c). (d-f), vascular cast after MCAO, showing areas of infarction where no blood vessels are visible (d-e). The regular pattern of micro-vasculature is also lost in the vicinity of the infarction. In 'f', apparently stressed micro-vessels are visible 24h after stroke (arrow). (g-j) Three days after MCAO, the first vascular buds are visible at many sites (arrows). The smallest micro-vessels formed connections with the surrounding proliferating vessels (h-j). In animals perfused for 2 weeks after MCAO, some micro-vessels had collapsed close to the budding vessels, suggesting that no-reflow phenomenon may have occurred (k). Both in the cortical regions and deeply within the brain vasculature, small micro-vessels formed very dense nests of proliferation usually around larger micro-vessels (i). These conglomerates increased in size with time of survival (see reference [66]).

kinases, in penumbra associated EC following MCAO in a rat model. Selected over-expression of these proteins might be involved in cellular survival and revascularization [12, 62].

Endothelial cell apoptosis

EC apoptosis can occur in response to the hypoxic conditions associated with stroke (Fig. 11). Oxygen-glucose deprivation induces iNOS expression, thereby increasing concentrations of nitric oxide and peroxynitrites associated with apoptosis [89]. The inflammation-associated increase in TNF-α expression, was accompanied by an increased NO expression in murine vascular EC through an undetermined mechanism [90], and also induced expression of reactive oxygen species through the Rho family GTP binding protein Rac1, and during reperfusion through a pathway incorporating PKC [91, 92]. Excitotoxicity induced mitochondrial damage may result in activation of caspase 9, and initiate apoptosis by the

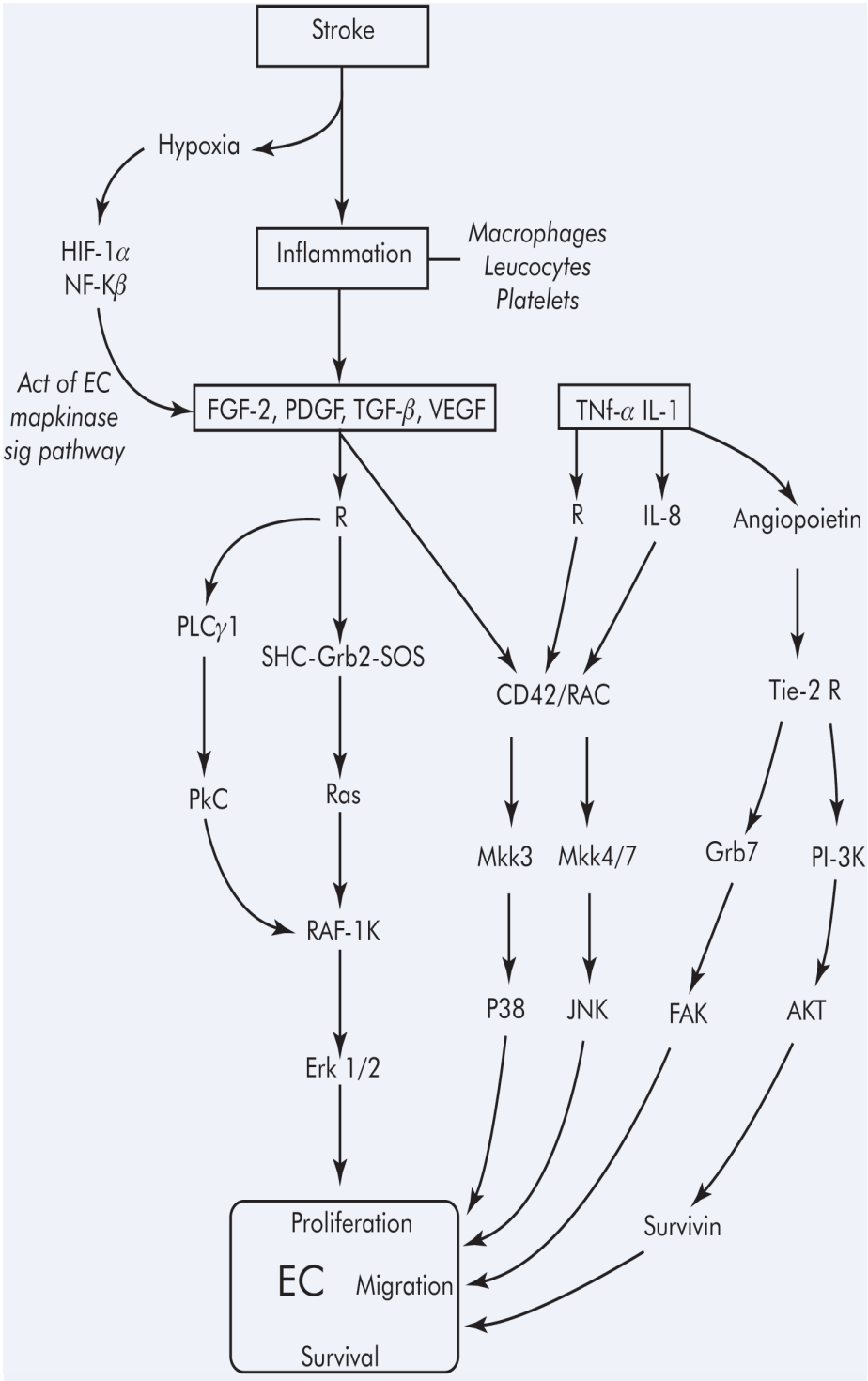

Fig. 10 Possible mechanisms of angiogenesis following acute ischaemic stroke. Growth factors including FGF-2, PDGF, VEGF and TGF-β are released during inflammation and hypoxia by brain parenchymal and immune cells. These angiogenic factors promote EC proliferation through conventional ras-ERK1/2 pathways and migration *via* CD42-p38/JNK. Inflammatory cytokines such as TNF-α and IL-8 can also activate p38 and JNK pathways. Furthermore, cytokine-induced expression of angiopoietin, can stimulate EC migration through FAK and survival through PI-3K-AKT. Abbreviations: HIF-1α, hypoxia-inducible factor-1 alpha; NF-κB, nuclear factor kappa-B; FGF-2, fibroblast growth factor-2; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-beta; VEGF, vascular endothelial cell growth factor; PLC-γ, phospholipase C-gamma; PKC, protein kinase C, Grb2, growth factor receptor-bound protein-2; SOS, son of sevenless; ERK, early response kinase; MKK5, map kinase kinase-5; JNK, c-jun N-termimal kinase; IL, interleukin; PI-3K, phosphoinositol-3 kinase; FAK, focal adhesion kinase.

same mechanisms as for neurones [93]. Mediators of apoptosis and their associated second messenger pathways have not been fully described after stroke. Increased expression of JNK and p38 during hypoxia-reoxygenation in human cerebral microvessel EC, was associated with activation of caspase-3 and apoptosis [94]. Other mechanisms for inducing apoptosis exist. For example, hydrogen peroxide induced apoptosis in pulmonary vascular EC through apoptosis signal-regulating kinase (ASK-1) mediated activation of JNK and p38 [95]. The localization, mechanism of stimula-

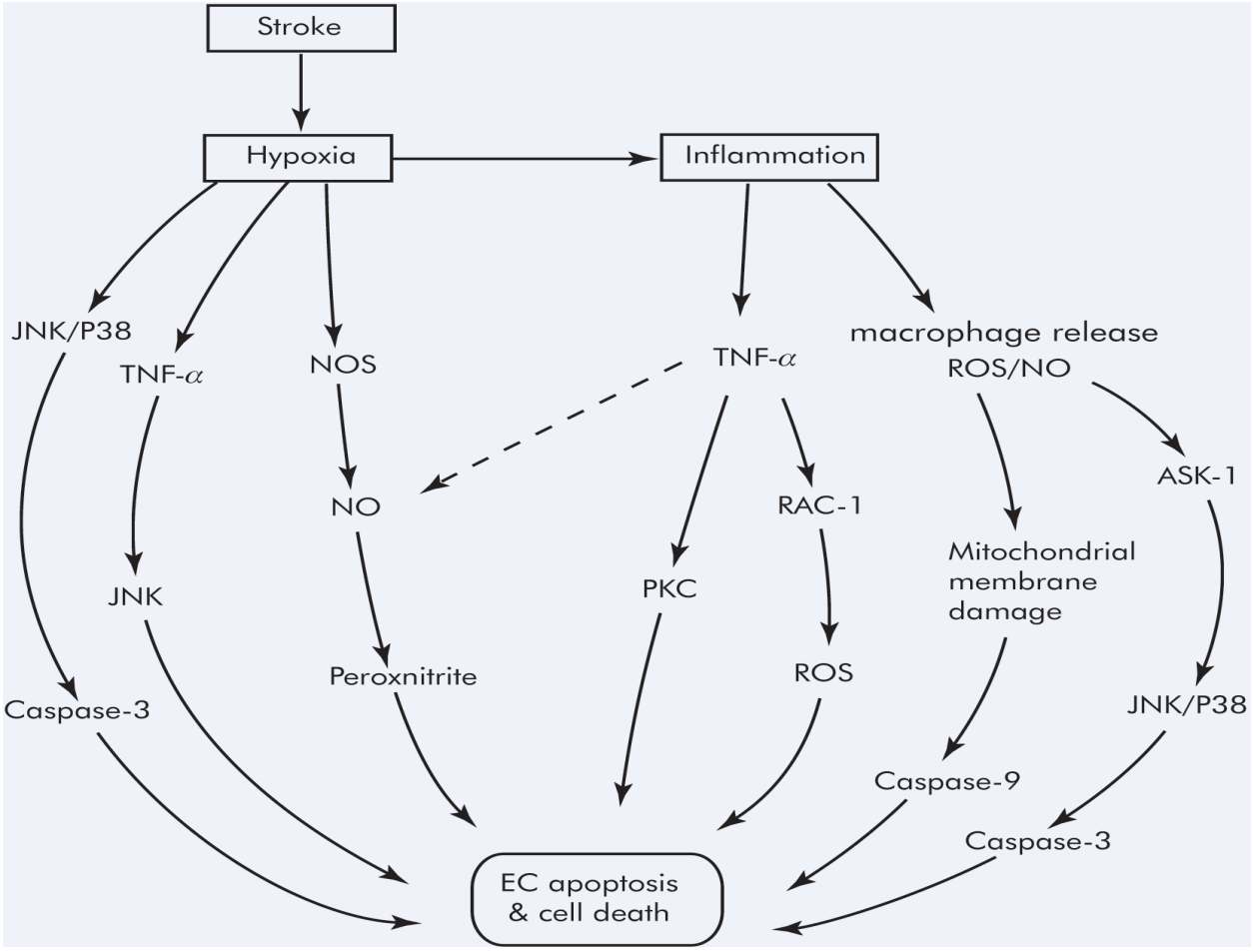

Fig. 11 Pathways of EC apoptosis following acute ischaemic stroke. The mechanisms through which EC undergo apoptosis following infarct have not been described in detail. *In vitro* data suggests stimulation of p38 and JNK MAP kinase pathways can induce activation of caspase-3 and stimulate EC apoptosis following hypoxia and in the presence of ROS. Increased expression of ROS and NO following stroke can lead directly to mitochondrial damage and further activation of the pro-apoptotic caspase cascade. Abbreviations TNF-α tumour necrosis factor-alpha; JNK, c-jun N-termimal kinase; NOS, nitric oxide synthase; NO, nitric oxide; PKC, protein kinase C; ROS, reactive oxygen species.

tion and time-course of activation of MAP kinases may be critical in determining the effect on survival and growth.

Several growth factors and associated signalling pathways have been identified in human stroke. Enhanced expression of the growth factors like VEGF [10, 62, 96], PDGF- β [63], TGF- β [97] and FGF-2 [Issa *et al*, unpublished data] has been demonstrated in penumbra tissue undergoing angiogenesis, suggesting that these factors might indirectly be determinants of neuronal survival after stroke. Increased phosphorylation of ERK1/2 has been reported to be localized around blood vessels in the penumbra, associated with an increased expression of VEGF and tyrosine phos-

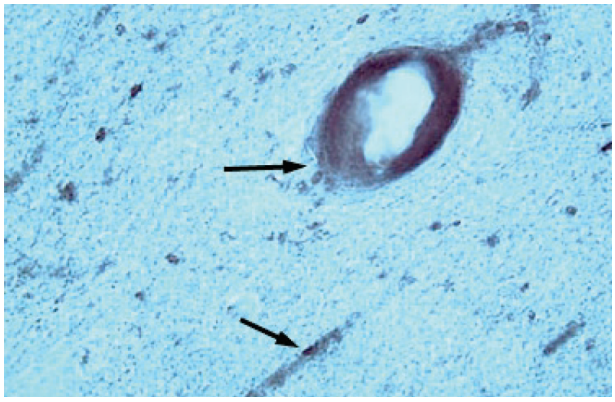

Fig. 12 Micro-vascular EC in penumbra tissue strongly stained in a patient who survived 4 days following acute ischaemic stroke (see reference [62]).

phorylated proteins in patients after stroke [62] (Fig. 12). VEGF may warrant further clinical investigation, since it was recently shown to enhance neuroprotection, neurogenesis and angiogenesis after MCAO in a rat model of stroke [76]. Preclinical studies have demonstrated both a reduction in stroke size, and recovery of sensorimotor function of impaired limbs after administration of FGF-2, and clinical trials of its intravenous administration as a cytoprotective agent in acute stroke have been performed [98]. Hepatocyte growth factor was strongly angiogenic, significantly reduced neurological deficit, and at the same time, did not induce cerebral oedema after gene transfection prior to MCAO in rat [99], suggesting its relevance to the progression of stroke.

Conclusions

A number of studies have examined gene regulation after ischaemic stroke in an animal model, using cDNA printed microarrays [100, 101], and have demonstrating deregulation of numerous novel genes. We have recently conducted a detailed series of microarray studies comparing global gene regulation in brain tissue following acute large vessel ischaemic stroke in patients surviving for 2 days-7 weeks, and following MCAO in a rat model, 1hour-21 days after stroke. The unpublished results showed notable differences in gene expression and time of expression between the human disease and the animal model. These studies suggest there is a need to determine the time course of expression of neuro-regulatory, angiogenic and EC apoptotic factors and their associated enzymes after ischaemic stroke in man. Non-therapeutically stimulated angiogenesis occurs only 3-4 days after stroke, which is beyond the period of reversible changes in ischaemic penumbra, recognised as a therapeutic window in ischaemic brain. Owing to the complexity of physiological regulation of blood vessel formation, involving numerous critical growth factors expressed differentially in time, space and concentration, ongoing therapeutic efforts using single agents, and aimed at treatment of vascular ischaemic disease are of limited potential. Growth factor induced signal transduction *e.g. via* JNK and p38 MAP kinase stimulates EC growth and might be beneficial. The same factors may stimulate apoptosis in neurons. It is likely that optimum conditions for angiogenesis together with subsequent neuronal protection may require therapeutic, cell specific, modulation of these intermediates, together with their stimulating factors.

Optimization of therapeutic treatments might involve a complex series of interventions beginning with self-treatment to reduce excitotoxicity-associated cell death within minutes of infarction. A cocktail of drugs could then be administered within the first few hours of illness to reduce inflammation, but at the same time, maintain neuronal viability with neurotrophins and stimulate growth factor-induced angiogenesis. Perfusion pressure in the penumbra region could be increased using thrombolytic therapy and susceptible neurons could be protected from apoptosis by viral transfer of genes such as Bcl-2 [102, 103]. In the recent past, progress in the ability to transfer proteins by protein transduction technology across the blood-brain barrier, as well as advances in neurological gene therapy, which has shown that brain defects in experimental disease models can be prevented and corrected [104, 105], indicates that sufficient information is now available to contemplate radical changes in treatment strategies in patients with stroke.

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