J. Cell. Mol. Med. Vol 15, No 8, 2011 pp. 1645-1687

Growth factors in ischemic stroke

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Received: July 7, 2009; Accepted: November 26, 2009

Abstract

Data from pre-clinical and clinical studies provide evidence that colony-stimulating factors (CSFs) and other growth factors (GFs) can improve stroke outcome by reducing stroke damage through their anti-apoptotic and anti-inflammatory effects, and by promoting angiogenesis and neurogenesis. This review provides a critical and up-to-date literature review on CSF use in stroke. We searched for experimental and clinical studies on haemopoietic GFs such as granulocyte CSF, erythropoietin, granulocyte-macrophage colony-stimulating factor, stem cell factor (SCF), vascular endothelial GF, stromal cell-derived factor-1α and SCF in ischemic stroke. We also considered studies on insulin-like growth factor-1 and neurotrophins. Despite promising results from animal models, the lack of data in human beings hampers efficacy assessments of GFs on stroke outcome. We provide a comprehensive and critical view of the present knowledge about GFs and stroke, and an overview of ongoing and future prospects.

Keywords: stroke • growth factors • G-CSF • EPO • GM-CSF • SCF • VEGF • IGF-1 • SDF-1α

Background

Stroke is a leading cause of death and disability worldwide among the adult population. Despite the recognised efficacy of some interventions in the acute phase, the narrow therapeutic window makes these treatments applicable to only a minority of stroke

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doi:10.1111/j.1582-4934.2009.00987.x

patients. Once the infarct is established, no therapy can help recover or replace the lost tissue, and rehabilitation and pharmacological treatment of co-morbidities are the only therapeutic strategies remaining for most stroke patients.

Thus, new approaches to restore function after stroke are under investigation. Some evidence from pre-clinical studies suggests that stem cell transplantation may represent a potential option for stroke treatment. However, several limitations, including experimental conditions for the *in vitro* culture of neural stem cells, choice of stem cell type, best route of administration and establishment of neuronal connections make it difficult to apply this treatment in a clinical setting [1, 2]. Moreover, since cell transplantation also requires surgical intervention in some cases, it is desirable to explore less invasive therapeutic strategies. The results of the first pre-clinical and clinical studies on stem cell transplantation highlight that cell differentiation, survival and trophic cell support is promoted by growth factors (GFs). In endogenous neurogenesis, GFs induced proliferation and differentiation of adult neural stem cells from the sub-ventricular zone of lateral ventricles (SVZ) and the dentate gyrus (DG) of the hippocampus into mature neurons in both animal models [3–6] and human studies [7, 8]. These findings suggest that GF therapy could represent an alternative therapeutic approach to promote migration and differentiation and to enhance the survival of endogenous stem cells by modulating pathways of endogenous neurogenesis.

Experimental studies show that hematopoietic GFs can improve stroke outcome through their pleiotropic effects which include neuroprotection, stem cell survival and promotion of angiogenesis and neurogenesis, as well as through their anti-apoptotic and anti-inflammatory effects [9].

Most GFs act binding specific receptors activating different signalling pathways and inducing the expression of specific genes involved in cell proliferation and differentiation. For instance, receptor-mediated activation of the mitogen-activated protein kinase (MAPK) promotes proliferation [10], whereas stimulation of the phosphoinosotide-dependent kinase/Akt pathway induces differentiation of stem cells. Thus, the response to specific GFs is influenced by the expression of their corresponding receptors on target cells [11, 12].

This review provides a critical, up-to-date evaluation of the literature relevant to the role of select GFs in post-stroke recovery. Both experimental models and human studies of ischemic stroke are included and discussed.

Search strategies

We included studies (abstracts, letters, articles, case–control studies, reviews and meta-analyses) on experimental models of stroke and in human beings. Our search focused on the GFs most often studied in ischemic stroke. The literature search included articles from 1960 to October 2009 in electronic bibliographic databases (MEDLINE, EMBASE). Reference lists from primary and review articles, and the MEDLINE function 'related articles' were also consulted. As search key words, we used: granulocyte colony-stimulating factor (G-CSF), erythropoietin (EPO), granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), vascular endothelial growth factor (VEGF), stromal cell-derived factor-1α (SDF-1α) and insulin growth factor-1 (IGF-1), and ischemic stroke, stroke, cerebrovascular disease and stem cell transplantation. Finally, we also examined non-English articles and studies on haemorrhagic stroke.

Haematopoietic growth factors

Red cells, granulocytes, monocytes, platelets and lymphocytes all derive from a common multipotent bone-marrow stem cell. Haemopoietic GFs, also known as CSFs, modulate lineage-specific differentiation of bone marrow stem cells (BMSCs), leading to the generation of circulating red cells, white cells and platelets.

Data from experimental studies (Table 1) support the notion that CSFs could improve stroke outcome by reducing stroke damage and improving post-stroke brain repair [9].

Granulocyte colony-stimulating factor

Human G-CSF is a 19.6 kD glycoprotein encoded by a single gene located on chromosome 17q11–12 [13]. G-CSF contains a hydrophilic signal sequence [14], which is responsible for the mobilization and proliferation of BMSCs, leading to their differentiation in circulating neutrophilic granulocytes. Two variant forms of this protein can be derived from differential splicing of the G-CSF mRNA [15]. G-CSF is produced, above all, by activated monocytes in response to inflammatory cytokines [16], but also by endothelial cells, fibroblasts, mesothelial cells and platelets [17].

Tumour necrosis factor (TNF)-α, interleukin (IL)-1, GM-CSF, IL-4 and bacterial lipopolysaccharide could induce G-CSF production *in vitro* [18–22]. G-CSF functions as a stimulating factor after binding to its receptor (GCSF-R), which is a type I membrane protein consisting of four different domains; an immunoglobulin-like domain, a cytokine receptor-homologous domain and three fibronectin type III domains in the extracellular region [23]. G-CSFR is expressed not only by haematopoietic cells (neutrophils, platelets, lymphocytes and monocytes), but also by endothelial cells, neurons and glial cells [24–28]. GCSF-R activates a variety of intracellular pathways, including the Janus kinase (JAK)/signal transducer and activator of transcription (STAT), the Ras/MAPK and phosphatidylinositol 3-kinase (PI3K)/protein kinase B [29–32]. Activation of these pathways mediates proliferation, differentiation and survival of hematopoietic cells [33].

The pharmacological effects and side effect profile of G-CSF are well known, since recombinant G-CSF is a licensed drug commonly used in clinical practice to treat chemotherapy-induced neutropenia and to boost stem cell mobilization in bone-marrow transplantation. The most common side effects are bone pain and

Table 1 Growth factors experimental studies

Growth factors	Study reference	Animal model	Sample size (treatment group/control group)	Type of study (focal or global ischemia)	Treatment	Time of administration	Results
G-CSF	[51]	Rat	12/12	90-min. MCAO	GCS-F 60 mcg/kg i.v. within 24 hrs	30 min. after the induction of ischemia	47% reduction of infarct volume (132.0 ± 112.7 mm^3 in the G-CSF group *versus* 278.9 ± 91.6 mm^3 in the vehicle group). Up-regulation of STAT3 in the peri-ischemic area.
	[52]	Mouse	12/15	60-min. MCAO	GCS-F 50 mcg/kg s.c. within 24 hrs or vehicle	24 hrs after occlusion	Reduction of infarct size (27 ± 7 mm^3; n = 9 in the G-CSF group *versus* 69 ± 5 mm^3; n = 3 in the vehicle group). Significant increase in survival rate (75% *versus* 20% in the treatment group).
	[68]	Rat	15/15	60-min. MCAO	GCS-F 50 mcg/kg s.c. within 24 hrs or vehicle	24 hrs after occlusion	Infarct volume reduction (61 ± 12 mm^3 in G-CSF–treated animals *versus* 176 ± 20 mm^3 in the vehicle group). Significantly increased numbers of $BrdU^+$ cells in their ipsilateral hemispheres in the treatment *versus* control group. Improved neurological behavior.
	[53]	Mice	12/15+6 (sham-operated)	60-min. MCAO	G-CSF injected s.c. (50 mcg/kg) or vehicle	1 hr after MCAO	46% reduction of infarct size (14.91 ± 3.5 mm^3 in the G-CSF group *versus* 27.66 ± 8.79 mm^3 in the vehicle group). Significant improvement in motor task.
	[63]	Rat	139/67	90-min. MCAO	G-CSF 50 mcg/kg i.p. in first 24 hrs (total of 150 mcg/kg)	2, 24, 96 or 168 hrs after occlusion	Reduction in infarct volume (106.9 ± 59.6 mm^3 in the treatment group *versus* controls 222.9 ± 80.4 mm^3), in hemispheric atrophy at 35 days and in BBB damage. Increased fraction of $BrdU^+$ endothelial cells. Significant increase in endothelial cells proliferation and in mean diameter of the cerebral microvessels in the ipsilateral hemisphere. eNOS and Ang2 up-regulation, Improved functional recovery.
	[36]	Rat	15/15	90-min. MCAO Direct distal MCAO Photothrombotic induction of ischemia	G-CSF 60 mcg/kg i.v. within 24 hrs G-CSF 60 mcg/kg i.v. within 24 hrs G-CSF 15 mcg/kg i.v. for 5 days	2 hrs after occlusion 1 hr after occlusion	Infarct size reduction (15% *versus* 37%). Increased recruitment of neural progenitors. Improved functional outcome after cerebral ischemia.
	[38]	Mice	35/35	60-min. MCAO	Recombinant human G-CSF (50 mcg/kg) intravascularly or vehicle	30 min. after vessel occlusion	Infarct size reduction (18.84 ± 3.72 mm^3 in the treatment group *versus* 28.59 ± 3.26 mm^3 in the control group). Increase in a time-dependent manner STAT3 expression. Stronger Bcl-2 and iNOS expression in the transition area in the G-CSF group.

Continued

Table 1 Continued

Growth factors	Study reference	Animal model	Sample size (treatment group/control group)	Type of study (focal or global ischemia)	Treatment	Time of administration	Results
	[54]	Rat		90-min. MCAO Photothrombotic induction of ischemia in the sensori-motor cortex.	GCS-F 60 mcg/kg i.v. within 24 hrs G-CSF 10 mcg/kg i.v	4 hrs after occlusion 1, 24 and 72 hrs after ischemia for 10 days.	33% infarct size reduction (223.33 $\pm$ 27.3 mm^3 in the G-CSF group *versus* 334.0 $\pm$ 31.5 mm^3 in the vehicle group). Improvement in sensorimotor deficits at the rotarod test.
	[39]	Rat	55	90-min. MCAO	Two groups: single dose of G-CSF s.c. (50 mcg/kg) or vehicle; G-CSF s.c. (50 mcg/kg) or vehicle for two additional days	At the onset of reperfusion or at the onset of reperfusion and for additional 3 days	Reduction of early neurological deficits, attenuation of infarct volume, increased neuronal and glial survival by activating different anti-apoptotic ways
	[55]	Rat	56/40	60-min. MCAO	G-CSF 10 mcg/kg s.c. within 24 hrs (total 50 mcg/kg)	6 hrs after occlusion	Non-significant infarct size reduction. Significant increase of the survival rate and of the neurological improvement. Increased number of $CD34^+$ cells in the marginal zone of the infarction at 7, 14 and 21 days after cerebral ischemia.
	[64]	Rat	5/5	90-min. MCAO	Single dose G-CSF injected s.c. (50 mcg/kg) or vehicle	With reperfusion	Increased number of $BrdU^+$ cells in the G-CSF-treated group.
	[65]	Rat	10/10	90-min. MCAO	G-CSF injected s.c. (50 mcg/kg) or vehicle	After reperfusion	31% reduction infarct volume 31% (25.1 $\pm$ 12.1% in the G-CSF group *versus* 56.5 $\pm$ 13.7% in the control group). Increased $BrdU^+$ cells at 28 days in the G-CSF group. Increased NeuN plus BrdU double-positive cells and in the G-CSF-group at 7 days. Increase in $BrdU^+$ endothelial cells in G-CSF-treated group (16.0 $\pm$ 4.5/mm^2) compared with vehicle-treated group (7.0 $\pm$ 2.0/mm^2), no longer persisting at 28 days.
	[60]	Rat	20/20+5	90-min. MCAO	G-CSF injected s.c. (50 mcg/kg) or vehicle	Immediately after riperfusion	55.6% reduction in infarct volume 119.4 $\pm$ 44.9 mm^3 in the G-CSF group *versus* 269.1 $\pm$ 42.1 mm^3 in the control group. G-CSF treatment improved the neurologic outcome by 50% at 24 hr. Significant reduction of TNF-a-positive cells at 8 to 72 hr and TGF-b2- and iNOS-positive cells at 24 and 72 hr after MCAO in the peri-ischemic area.

Continued

Table 1 Continued

Growth factors	Study reference	Animal model	Sample size (treatment group/control group)	Type of study (focal or global ischemia)	Treatment	Time of administration	Results
	[62]	CB-17 mice		MCAO	Human recombinant G-CSF s.c. (0.5, 5, 50 or 250 mcg/kg)/continuous administration (100 mcg/kg/day) by micro-osmotic pump or PBS or recombinant human EPO	At 24, 48 and 72 hrs after stroke or 1 hr after stroke (at doses of 0.5, 5, 50 or 250 mcg/kg) or 1 hr after stroke over 7 days	Negative effect on brain injury, brain atrophy and on functional recovery after stroke, enhancement of inflammatory response, positive effect on angiogenesis.
	[61]	BL/6J mice	13+11/33	MCAO	Recombinant human G-CSF 300 mcg/kg and recombinant murine SCF 100 mcg/kg or vehicle	Acute phase (days 1–10) and in the subacute phase (days 11–20 after the occlusion)	Increased expression of mRNA IL-10 and anti-inflammatory cytokines.
	[66]	Mice		Permanent MCAO after sex-mismatched bone marrow transplantation from EGFP-expressing mice	G-CSF/SCF treatment		G-CSF/SCF treatment reduced infarct volumes by more than 50%, resulted in a 1.5-fold increase in vessel formation, led to a 2-fold increase in the number of newborn cells in the ischemic hemisphere.
	[35]	Male Wistar rats	15	60 min. temporary MCAO	i.v. saline or G-CSF (60 mcg/kg)	30 min. after temporary MCAO	G-CSF significantly attenuated the release of glutamate in the infarcted striatum from 30 min. to 180 min. after tMCAO compared with controls ($P < 0.05$). Infarct volume reduced significantly compared to controls at 24 hr after tMCAO.
EPO	[110]	Rat		60 min. MCAO	i.p. administration of EPO (5,000 units/kg of body weight, i.p.)	At the time of occlusion	Reduction of the volume of infarction, almost complete reduction in the number of terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling of neurons within the ischemic penumbra.
	[118]	7 days old Rat		right CCAO	i.p. DFO (200 mg/kg), recombinant human EPO (1 kU/kg), a combination of DFO-EPO or vehicle	0, 24 and 48 hrs after hypoxia-ischemia	DFO-EPO administration reduced the number of cleaved caspase 3-positive cells in the ipsilateral cerebral cortex. However, treatment with DFO, EPO, or with the combination of DFO and EPO did not protect against grey or white matter damage in the experimental setting applied.

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Table 1 Continued

Growth factors	Study reference	Animal model	Sample size (treatment group/control group)	Type of study (focal or global ischemia)	Treatment	Time of administration	Results
	[120]	Rat		MCAO	rhEPO; 5,000 U/kg body weight, i.p.	at the time of MCA occlusion	significant reduction of astrocytes, leucocytes and microglia activation as well as a decreased production of pro-inflammatory cytochines such as TNF-α, IL-6 and monocite chemoattractant protein 1 in ischemic area
	[122]	Rat		Focal cerebral ischemia	i.v. treatment with CEPO	At different time points after MCA occlusion	Reduced cellular infiltration, reduced apoptosis and white matter damage
VEGF	[203]	Rat	7/10	MCAO by placement of placement of a clot	rhVEGF 1 mg/kg over a 4 hr interval (5 μl/min) or vehicle	1 hr or 48 hrs after embolization	Enhancement of angiogenesis in the ischemic brain and reduction of neurological deficits during stroke recovery
	[200]	Rat	6/5	90-min. MCAO	VEGF (1.0 ng/μl, 9 μl) was topically applied on the surface of the reperfused rat brain or vehicle	After reperfusion	Reduction in infarct volume (18.4% $\pm$ 2.1% in the VEGF group *versus* 33.2% $\pm$ 4.2% in the control group) and brain edema (81.5% $\pm$ 0.8% in the VEGF group *versus* 84.9% $\pm$ 2.1% in the vehicle treated group). Anti-apoptotic effect demonstrated by a significant decrease in TUNEL staining both at 24 (32.8 $\pm$ 8.5 cells/mm^2 *versus* 46.4 $\pm$ 7.2 cells/mm^2 in the control group) and 48 hr (40.7 $\pm$ 12.5 cells/mm^2 *versus* 68.1 $\pm$13.6 cells/mm^2 in the non-VEGF group).
	[207]	adult male CD-1 mice	Six groups of 6 adult male CD-1 mice underwent 1) AdlacZ (viral vector control), 2) AdVEGF, 3) AdAng2, 4) VEGF protein, 5) VEGF protein plus AdAng2, 6) saline (negative control) injection		2 mcL of adenoviral suspension (AdVEGF, AdAng2, or Ad*lacZ*) were injected stereotactically into the right caudate/putamen. For the protein infusion a i.c.v. infusion *via* osmotic minipump was used.		Mice treated with VEGF protein infusion plus AdAng-2 significantly increased microvessel counts relative to all other groups ($P < 0.05$). Combination of VEGF and Ang-2 may lead to BBB disruption because it increases MMP-9 activity and inhibits ZO-1 expression.

Continued

Table 1 Continued

Growth factors	Study reference	Animal model	Sample size (treatment group/control group)	Type of study (focal or global ischemia)	Treatment	Time of administration	Results
	[211]	Adult C57/BL 6 mice		Animals were exposed to normobaric hypoxia at 12 ± 6% oxygen for 24 hrs or were kept at room air pressure	A neutralizing goat antimouse VEGF antibody or a corresponding control antibody was injected i.p. at 100 mg	Immediately prior to the 24 hrs hypoxic or normoxic exposure.	Hypoxic exposure led to a significant increase in the levels of VEGF mRNA and protein in mouse brain that correlated with the severity of the hypoxic stimulus. Inhibition of VEGF activity by a neutralizing antibody completely blocked the hypoxia-induced increase in vascular permeability.
	[212]	Male Wistar rats	10 (VEGF antagonist treated)/15 (PBS treated)	2-VO model in which 2 adjacent cortical veins were photochemically occluded	i.p. injection	Immediately after 2-VO	In treated animals there were attenuated vascular permeability and reduced cortical venous infarct in the acute stage.
	[214]	Gerbil model of focal brain ischemia	24+24 treated/24+24 controls	A single 3-min. bilateral CCAO initially (about 4–5 min.)	Animals were treated in the left lateral cerebral ventricle with rAAV-LacZ (control) or with rAAV-VEGF (0.5–25 U).	Pre-treatment (6 days or 12 days)	Improved survival, brain edema and delayed neuronal death in treated animals.
	[205]	Rat	Group 1 (ischemia-only = 12), group 2 (ischemia + VEGF = 12), group 3 (ischemia + NSCs = 12), group 4 (ischemia + NSCs + VEGF = 12)	Intraluminal thread occlusion of MCA for 90 min.	i.v. administration of human neural stem cells (hNSCs: 5×10^6) and/or i.v. recombinant human VEGF [50 mcg/kg, 1 mcg/(kg min)].	hNSCs: 24 hrs after surgery VEGF: 48 hrs after surgery	Better functional recovery, reduced cerebral atrophy and increased vascular density without a significant effect on stem cells survival, in hNSC+VEGF combined treated animals compared to the other groups.
	[199]	Rat	5+6+6/5+7+6 controls	2-hr MCAO	Recombinant human VEGF 165 (5 mcg/ml), or vehicle was infused into the right lateral ventricle by osmotic minipump	1 week before MCAO	I.c.v. infusion of VEGF 165 decreases infarct volume and brain edema after temporary MCAO without a significant increase in CBF.
	[202]	Mice	15/10	90-min. MCAO	VEGF (8 ng i.c.v.) i.v. VEGF (15 ng/kg)	1 or 3 hrs after reperfusion	Decreased infarct volume and improvement in neurological disability score. Enhancement of infarct volume.

Continued

Table 1 Continued

Growth factors	Study reference	Animal model	Sample size (treatment group/control group)	Type of study (focal or global ischemia)	Treatment	Time of administration	Results
	[204]	Rat		90-min. MCAO	VEGF (10 μg/ml) was administered at 1 μl/hr i.c.v.	For 3 days	Improvement of both sensorimotor and cognitive functional outcome.
	[216]	Rat	3 animals per 10 groups	temporary MCAO	VEGF165 was administered intra-arterially for 7 days by miniosmotic pump: total dosages: 2 μg (low dose), 8 μg (intermediate dose), or 60 μg (high angiogenetic dose)	After MCAO	Non-angiogenic VEGF 165 treated brains showed preserved neuropil and reduced numbers of macrophages; ischemic brains treated by high angiogenic dose showed phagocytized neuropil and high macrophage density.
	[198]	Rat	144	30-min. MCAO	i.c.v. injection of 5 mcg human VEGF 165-expressive plasmid (phVEGF) mixed with liposome	At 15, 120, or 360 min. after ischemic operation	VEGF overproduction improves stroke-induced striatal neurogenesis and enhances their maturation
	[210]	Rat	14/14	120-min. MCAO	3×10^6 MSCs in 1 ml total fluid volume PBS or vehicle	24 hrs after MCAO	MSCs treatment promotes angiogenesis and vascular stabilization, which is at least partially mediated by VEGF/Flk1 and Ang1/Tie2.
	[220]	Rat	5+5/5	Transient MCAO	Intracerebral administration of VEGF gene-transferred BMSCs engineered with a replication-deficient HSV type 1 1764/4-/pR19-VEGF 165 vector or native BMSCs, or vehicle	24 hrs after MCAO	More potent autologous cell transplantation therapy than the transplantation of native BMSCs alone (significant functional recovery, lower infarct volume, stronger expression of VEGF proteins).
	[201]	Rat		90 min. of MCAO	Pretreatment with KDR kinase inhibitor (Compound-1) (40 mg/kg p.o.)	Starting 0.5 hr before occlusion	Early pre-treatment administration of a KDR kinase inhibitor elicited an early, transient decrease in edema and subsequent reduction in infarct volume, implicating VEGF as a mediator of stroke-related vascular permeability and ischemic injury.

Continued

Table 1 Continued

Growth factors	Study reference	Animal model	Sample size (treatment group/control group)	Type of study (focal or global ischemia)	Treatment	Time of administration	Results
	[218]	VEGF (hi/+) and VEGF (lo/+) mice		Femoral artery ligation MCA ligation			Compared to VEGF(hi/+) mice, VEGF(lo/+) formed fewer collaterals during the perinatal period when adult density was established, and had 2-fold larger infarctions after MCA ligation, suggesting that VEGF and VEGFR-1 are determinants of arteriogenesis. VEGF-A specifies formation of native collaterals in healthy tissues.
	[215]	Wistar rats	93	MCAO for 2 hrs	rAAV-VEGF, rAAV-null or physiologic saline was delivered into the lateral ventricle	8 weeks before MCAO	I.c.v. rAAV-VEGF pre-treatment results in deleterious intracranial hypertension and augments secondary ischemic insults at the early stage of MCAO.
	[219]	Rat	4 for each condition		NPCs expressing VEGF-A165 transplantation into the caudate putamen (1,000,000 cells)		Increased survival of adenovirally transfected NPCs after 11 days, but not after 24 hr or 4 days. Increased expression of the endothelial cell marker PECAM-1 (CD31) after 24 hrs, 4 days, and 11 days after transplantation.
	[213]	Rat			Stereotaxic injection of VEGF 1 mcg into the left SN		VEGF administration highly up-regulated AQP4 mRNA and protein in the ventral midbrain, localized in close proximity to the VEGF-induced new blood vessels.
	[217]	Squirrel monkeys	3/4	Bipolar electrocoagulation of cortical vessels supplying the M1 hand area representation			HIF-1a up-regulation is confined to the infarct and peri-infarct regions. Increases in VEGFR-2 immunoreactivity occurred in two remote regions.
GM-CSF	[162]	Rat	16/18	Bilateral vertebral and unilateral CCAO followed by hemodynamic stroke	Daily injections of GM-CSF or vehicle		Induction of arteriogenesis
	[157]	Rat		Combined CCA/distal MCAO with 180 min. occlusion followed by 72 hrs reperfusion or proximal MCAO with 90 min. occlusion and 72 hrs reperfusion	10 mg/kg bodyweight GM-CSF i.v. over a time period of 20 min. or vehicle		GM-CSF provides protection against experimental stroke and counteracts programmed cell death.

Continued

Table 1 Continued

Growth factors	Study reference	Animal model	Sample size (treatment group/control group)	Type of study (focal or global ischemia)	Treatment	Time of administration	Results
	[161]	Rat	13/8+8	Occlusion of vertebral plus left carotid artery	GM-CSF or vehicle applied intraoperatively by a single intra-arterial dose into the non-occluded CA and subsequently by s.c injection every day		GM-CSF treatment did not influence the arterial angioarchitecture on the dorsal brain surface but increased vessel diameter during natural arteriogenesis
	[155]	Rat	25/23	1 hr intraluminal MCAO	Intracarotid injection of GM-CSF (5 ng) or saline	Immediately after reperfusion	Reduced the infarct volume and improved neurological function at 48 hrs after reperfusion in treated animals. Increased number of activated microglia/macrophages and decreased number of apoptotic cells in the penumbra area.
	[163]	Rat	Not known	BCAO	BCAO and daily subcutaneous injection with GM-CSF 10 mcg/kg diluted in 0.3 ml saline, or vehicle, or sham occlusion and vehicle	Injection started with occlusion of the first carotid artery until 5 weeks after the second CAO	CBF and cerebrovascular reserve capacity recovered completely in GM-CSF–treated animals but not in solvent-treated animals. The capillary density showed a mild increase in GM-CSF–treated animals. The number of intraparenchymal and leptomeningeal arterioles was significantly higher in GM-CSF–treated animals than in both other groups.
	[160]	Rat model of postnatal hypoxia–ischemia	Hypoxia–ischemia (HI, $n = 67$), hypoxia–ischemia with G-CSF treatment (HI+G, $n = 65$), healthy control (C, $n = 53$).	Right CCAligation	G-CSF (50 μg/kg s.c.)	Start 1 hr after HI and given on 4 subsequent days	Improved quantitative brain weight and qualitative Nissl histology. Decreased apoptotic cells (TUNEL positive), with reduced expression of Bax, cleaved caspase-3 and with increased expression of STAT3, Bcl-2, and Pim-1 in HI+G treated animals.
	[158]	Adult male C57BL/6 strain mice		Unilateral CAAO	Injection of GM-CSF (20 mcg/kg s.c.) or saline injection	Every second day after CCAO until 7 days after CCA0	GM-CSF treatment produced increased leptomeningeal collateral growth, an increase in the number of Mac-2+ monocytes/macrophages on the surface of the brain and decreased infarct size
	[159]			unilateral MCAO	GM-CSF at 60 mug/kg	Daily for 5 consecutive days beginning immediately after injury	GM-CSF leaded to decrease the extent of neuronal apoptosis by modulating the expression of several apoptosis-related genes such as Bcl-2, Bax, caspase 3, and p53, resulting in decreased infarction volume and improved locomotor behavior.

Continued

Table 1 Continued

Growth factors	Study reference	Animal model	Sample size (treatment group/ control group)	Type of study (focal or global ischemia)	Treatment	Time of administration	Results
SCF	[59]	GFP-bone marrow engrafted mice (into the tail vein after irradiation)	6 for each treated group 6 for each control group	Permanent MCAO	300 mcg/kg of recombinant human G-CSF and/or 100 mcg/kg of recombinant murine SCF. BrdU (50 mg/kg per day, s.c.)	Cytokines: in the acute phase (days 1 to 10) or subacute phase (days 10 to 20 days) after MCAO BrdU: together with cytokines for 2 days, and 24 hrs after the last injection	Subacute phase treated animals improved motor performance and higher brain function, compared with acute-phase treatment. Acute-phase and subacute-phase treatments identically reduced the infarct volume relative to vehicle treatment. Subacute-phase treatment significantly induced transition of bone marrow derived neuronal cells into the peri-infarct area and stimulated proliferation of intrinsic neural stem/progenitor cells in the neuroproliferative zone.
	[260]	Rat	Saline, SCF, G-CSF, and SCF+G-CSF (n = 10/group)	Permanent right MCA ligation	Recombinant rat SCF (200 mcg/kg) and/or recombinant human G-CSF (50 mcg/kg) s.c.	Start at 14 weeks after brain ischemia, for 7 days.	Significant functional improvement was seen in SCF+G-CSF-treated rats 1, 5, and 17 weeks after injections. SCF alone also improved but not stable functional outcome. No functional benefit was seen in G-CSF-treated rats. Infarction volume was significantly reduced in SCF+G-CSF-treated rats.
	[261]	Rat	1° experiment: saline control, SCF, G-CSF, and SCF+G-CSF (n = 10), Sham-operative control rats (n = 6) 2° experiment: saline, SCF, G-CSF, or SCF+G-CSF (n = 4)	Permanent right MCA ligation	1° experiment: s.c. injection of saline or GFs (recombinant rat SCF, 200 mcg/kg; recombinant human G-CSF, 50 mcg/kg). 2° experiment: s.c. injection of GFs plus BrdU; 50 mg/kg per day, i.p.	1°-2° experiments: 3 hrs to 7 days after brain ischemia.	SCF-treated rats showed the best functional restoration at 1, 4, 7 and 10 weeks after the final injection. G-CSF-induced limited and unstable functional recovery. Stable but delayed functional improvement in SCF+G-CSF-treated rats. Infarction size was significantly reduced in all GF-treated rats. SCF and SCF+G-CSF enhanced NPC proliferation in the sub-ventricular zone bilaterally, whereas G-CSF and SCF+G-CSF treatment increased – $BrdU^+$ cells in peri-infarct area.
	[320]		Group A, n = 13: i.c.v. treated with IGF-I; Group B, = 14: placebo; Group D, = 10: s.b. treated with IGF-I; Group E, = 10: placebo; Groups C and F sham-operated controls (= 5 and = 3, respectively).	Transient right MCAO for 1 hr	I.c.v.: 33.33 mcg IGF-I/d for 3 days, s.c.: 200 mg IGF-I/d for 7 days	Treatment was begun 30 min. after MCAO	There was less neurological deficit after ischemia in i.c.v. and s.c. IGF-I–treated animals compared with controls. Continuous treatment with i.c.v. and s.c. administered IGF-I achieved a long lasting neuroprotective effect as early as 24 hrs after ischemia.

Continued

Table 1 Continued

Growth factors	Study reference	Animal model	Sample size (treatment group/ control group)	Type of study (focal or global ischemia)	Treatment	Time of administration	Results
SDF-1-alfa	[255]	Rat	16/16	right MCAO ligation and BCCAO	Stereotaxical injections with recombinant human SDF-1alfa (4 mcg per one cortical area), vehicle, or control protein (BSA) into 3 cortical areas adjacent to the right MCA	At 30 min. after MCA ligation	Treated animals showed less cerebral infarction due to up-regulation of antiapoptotic proteins, and they had improved motor performance. SDF-1 alfa injection enhanced the targeting of bone derived cells to the injured brain, as demonstrated in GFP-chimeric mice with cerebral ischemia. Increased vascular density in the ischemic cortex of SDF-1alfa-treated rats enhanced functional local CBF.
	[256]	Rat and mice	From 6 to 12 for different conditions	Right MCA ligation and BCCAO	Intracerebral injection of human OECs/olfactory nerve fibroblasts (hOECs/ONFs) (1 $\times$ 106 cells) into 3 cortical areas adjacent to the right MCA, or vehicle.	1 day after brain ischemia. Cyclosporin A (CsA; 1 g/kg/d, i.p.) injections were given daily to each experimental rat from the day after cerebral ischemia for 3 weeks	Transplanted hOECs/ONFs and endogenous homing stem cells colocalized with specific neural and vascular markers, indicating stem cell fusion. Both hOECs/ONFs and endogenous homing stem cells enhanced neuroplasticity in the rat and mouse ischemic brain. The up-regulation of SDF-1α and the enhancement of CXCR4 and PrPC interaction induced by hOEC/ONF implantation mediated neuroplastic signals in response
	[246]	Male virus- and pathogen-free NMRI mice	27 (comprensive of controls)	MCAO			After focal cerebral ischemia, SDF-1alfa is up-regulated in the peri-infarct and infarct tissue and this up-regulation was accompanied spatially and temporally by CXCR4-expressing infiltrates.
	[247]	Mice	40	MCAO	Mice received bone marrow transplants from GFP transgenic donors and later underwent a MCAO.		SDF-1 expression was detected in the infarcted hemisphere within 24 hr and it is maintained through at least 30 days after MCAO. It was princip ally localized to the ischemic penumbra. At 14 days post occlusion an association between transplanted bone marrow derived cell location and density and the level of SDF-1 immunoreactivity. These findings suggest that SDF-1 has an important role in the homing of bone marrow derived cells, to areas of ischemic injury.
	[242]	Female Rat	25	2-hr MCAO	3 $\times$ 106 male rat BMSCs	1 month after MCAO	Treated animals showed significant recovery of behaviour. Expression of SDF-1 was significantly increased along the ischemic boundary zone compared with the corresponding areas in the contralateral hemisphere, suggesting the role of interaction of SDF-1/CXCR4 on the trafficking of transplanted BMSCs.

Continued

Table 1 Continued

Growth factors	Study reference	Animal model	Sample size (treatment group/ control group)	Type of study (focal or global ischemia)	Treatment	Time of administration	Results
	[257]	Rat	Naive rats and rats 6 hrs, 1, 2, 4 and 10 days after surgery (n = 3 to 5).	Permanent unilateral MCAO or sham operation			A strong increase of SDF-1 and CXCR4 expression in infarct and peri-infarct region, 2–10 days after stroke was reported.

MCAO: middle cerebral artery occlusion; i.v.: intravenously; s.c.: subcutaneously; i.p.: intraperitoneally; BBB: blood brain barrier; CCAO: common carotid artery occlusion; SVZ: sub-ventricular zone; i.c.v.: intracerebroventricularly; NSCs: neural stem cells; BMSCs: bone mesenchimal stem cells; BCAO: bilateral carotid artery occlusion; NPCs: neural progenitor cells; CBF: cortical blood flow; BCCAO: bilateral common carotid artery occlusion; SN: substantia nigra; DFO: Deferoxamine; CEPO: non-erythropoietic EPO derivate.

asymptomatic increases in serum alkaline phosphatase and γ-glutamyl trans-peptidase [34].

G-CSF and experimental stroke

In vitro, G-CSF was reported as being neuroprotective in cell cultures exposed to glutamate-induced excitotoxicity [35] and to promote neuronal differentiation [36] G-CSF was identified as having trophic effects on different cell types within the central nervous system (CNS) and to induce neuroprotection during recovery after brain injury [37]. Endogenous G-CSF seems to play an important role in the brain's response to ischemia. G-CSF and its receptor are expressed in response to ischemia in the penumbral region of ischemic stroke [36] where it has an anti-apoptotic effect, resulting in reduced cell death in the ischemic penumbra [36, 38, 39]. Data from a post-mortem study demonstrated a strong neuronal G-CSF and GCSF-R immunoreactivity in the infarct and peri-infarct areas in both acute and subacute stroke. Neuronal G-CSF was expressed as a very early response to ischemic stroke, whereas in the subacute phase it was predominantly up-regulated in vascular cells [40]. A clear demonstration of the role of endogenous G-CSF in response to brain ischemia comes from the detection that G-CSF-deficient mice are prone to develop larger infarcts and have worse clinical outcome, and that these effects were reversed after G-CSF administration. In the same study, both real time PCR and immunohistochemistry detected an up-regulation of matrix metalloproteinase 9 (MMP-9), proteins that are normally present in the brain in latent form and that, once activated, contribute to the injury process through neurovascular matrix degradation and increased vascular permeability [41]. This finding also supports a role for GF in the endogenous response to ischemic brain damage [42–44].

In an animal model of permanent as well as transient middle cerebral artery occlusion (MCAO), G-CSF produced a significant reduction in brain oedema partly mediated by suppression of injury-induced up-regulation of IL-1, TNF-α and endothelial nitric oxide synthase (eNOS) mRNA [45]. Blood brain barrier (BBB) damage is probably prevented by the decreased production of pro-inflammatory cytokines, the resulting T-cell infiltration [46, 47], and by up-regulation of anti-inflammatory molecules [48] that may be involved in the neuroprotective activity. In addition, G-CSF mobilizes exogenous $CD34^+$ stem cells and promotes their homing to the site of injury in rat brain [49, 50]. After focal cerebral ischemia, G-CSF administration significantly reduced infarct size, mortality and improved neurological function [51, 55].

Two recently published meta-analyses confirm these results. Minnerup *et al.* reviewed 13 studies evaluating the efficacy of G-CSF in animal models with focal cerebral ischemia. In a total of 277 animals used to determine infarct size and 258 animals used for functional outcome assessments, an overall infarct size reduction of 42% and a functional improvement of sensorimotor deficits ranging from 24% to 40% were observed [56]. G-CSF efficacy in the acute phase (within 6 hrs) after stroke seemed to be dose dependent, and its beneficial effect after delayed administration was confirmed [56]. England *et al.* [57] included 666 animals from 19 different studies; all these studies, except for those by Lan *et al.* [58] and Han *et al.* [35], were included in the previous meta-analysis. Interestingly, the authors further analysed the outcome on the basis of different experimental models demonstrating a significant infarct size reduction after transient but not permanent ischemia [57].

Moreover, G-CSF administration after reperfusion in animal models of focal cerebral ischemia induced by transient (60–90 min.) MCAO was associated with a neuroprotective effect mediated by activation of anti-apoptotic pathways [38, 39, 59] and reduction of focal inflammatory responses [60, 61]. Indeed, phosphorylation of STAT3 and nuclear Pim-1 levels were significantly increased after G-CSF administration, and JAK2/STAT3 pathway activation may represent the anti-apoptotic effect of G-CSF

mediated through GCSF-R [36, 38, 39, 51]. Immunohistochemical analysis revealed a significant reduction in cells expressing pro-inflammatory cytokines such as TNF-α, transforming growth factor-β (TGF-β), and inducible nitric oxide synthase (iNOS), and an overexpression of the anti-inflammatory cytokine, IL-10 [61] in peri-ischemic areas [60].

In contrast to these data, a recent study in a murine model of cerebral ischemia showed that G-CSF induces an excessive inflammatory response, resulting in cortical atrophy and impaired behavioural function [62]. There is some evidence that, under ischemic conditions, G-CSF administration results in a significantly increased number of $BrdU^+$ endothelial cells during the acute phase [63] and after 7 days [64, 65]. Also, the combined administration of G-CSF and SCF increased the number of bone marrow-derived endothelial cells in mouse brain after cerebral ischemia [66]. G-CSF may also enhance proliferation and GF production in cultured BMSCs [67]. Taken together, these findings demonstrate that G-CSF promotes both neurogenesis and angiogenesis in peri-ischemic areas. Finally, G-CSF was shown to promote cell proliferation from SGZ of DG, leading to their mobilization and homing to brain [65, 68].

G-CSF and human stroke

The promising results in animal models of stroke led to the rationale for the clinical use of G-CSF, and phase I/II clinical trials were implemented to confirm whether similar responses could be obtained in ischemic human brains. We found three completed trials of G-CSF. The Stem cell Trial of Recovery Enhancement after Stroke was a randomized, controlled, pilot study to assess the safety of G-CSF administration in 36 patients with recent ischemic stroke and to evaluate its effect on circulating $CD34^+$ stem cells. Patients were recruited within 7 to 30 days from symptom onset and then randomized to receive either subcutaneous recombinant human G-CSF or placebo (saline) in a dose-escalation study. The primary outcome was peak circulating blood $CD34^+$ count. Safety assessments included mortality and clinical evaluations with the stroke scales score. This pilot study provided evidence that G-CSF administration in stroke patients is safe, well-tolerated and effective in mobilizing bone marrow $CD34^+$ stem cells, although the benefits on clinical outcome were uncertain [69]. Shyu *et al.* [70] assessed the safety and efficacy of G-CSF administration in patients with acute ischemic stroke in a small, randomized, blinded, controlled trial in Taiwan. The study was designed to investigate the tolerability and effectiveness of G-CSF administration in acute stroke patients. This trial included 10 patients with acute cerebral infarction (in the MCA territory confirmed by magnetic resonance imaging [MRI]), recruited within 7 days of symptom onset. The primary end-point was clinical improvement at the 1 year follow-up assessed through four different stroke scales; the National Institutes of Health (NIH) Stroke Scale, European Stroke Scale, ESS Motor Subscale (EMS) and Barthel index. As a secondary end-point, the authors used cerebral PET to quantify cerebral uptake of fluorodeoxyglucose (FDG) in the peri-infarct area.

Patients assigned to the treatment arm ($n = 7$) received 15 μg/kg G-CSF subcutaneously for five consecutive days. All patients underwent a 1–3 month follow-up visit for 1 year and FDG-positron emission tomography (PET) and MRI at 6 and 12 months. Despite the small sample size, this study supports the feasibility of subcutaneous (s.c.) G-CSF administration in stroke patients and suggests that G-CSF may reduce long-term disability. The benefit seemed to be greater in the subgroup of patients who underwent treatment within 24 hrs [70].

Zhang *et al.* [71] recruited 45 acute stroke patients to a randomized, placebo-controlled trial. Of these, 15 were randomized to recombinant G-CSF (rhG-CSF) 2 g/kg s.c. for 5 days within 30 days of symptoms onset, and 30 were enrolled in the control arm. The primary end-points were NIHSS score and adverse reactions. The authors found a significant reduction of NIHSS score in the treatment group on the 20th day of follow-up (this difference was absent in the placebo group). No adverse reactions were registered.

Recently, the Cochrane Systematic Review, including the three previous small clinical studies, failed to demonstrate a significant reduction in combined death and dependency in stroke patients after G-CSF administration, concluding that further studies are necessary to determine whether G-CSF administration could improve the outcome of stroke patients [72].

A multicenter, randomized, double-blind, placebo-controlled, dose-escalation phase IIA trial (AXIS) was recently completed. In the 45 acute stroke patients in this trial, G-CSF was safe, well-tolerated and showed clinical efficacy, even in patients with wide baseline lesions detected by diffusion-weighted imaging (DWI)-MRI [73].

Ongoing studies

A phase IIB/IIIA trial (AXIS), that will start in the second quarter of 2009 [74], and the Regeneration in Acute Ischemic Stroke Study, were implemented to confirm the safety of G-CSF administration in acute stroke patients [75]. The phase I, granulocyte colony-stimulating factor in ischemic stroke trial is also ongoing to verify the mortality and adverse effects of G-CSF therapy as the primary end-point (www.clinicaltrials.gov, NCT00809549). Another phase II clinical trial currently recruiting patients was implemented to assess whether s.c. G-CSF and erythropoietin administration (EPO) influence associative learning and/or motor skills in patients who experience chronic stroke or amyotrophic lateral sclerosis (www.clinicaltrials.gov, NCT00298597).

Current evidence and future perspectives

Endogenous G-CSF plays an important role in brain response to ischemia. G-CSF administration in animal models is associated with reduced infarct volume, decreased mortality and better clinical outcome. Recombinant G-CSF is already approved for limited clinical use in human beings. G-CSF administration in stroke patients was safe, but further studies should be implemented in order to demonstrate its efficacy.

Erythropoietin

EPO is a protein hormone of about 34.4 kD and a member of the class I cytokine family [76]. Human EPO is encoded by a single gene consisting of 5 exons located on chromosome 7 [77]. Gene expression is controlled by an oxygen-sensing, hypoxia-inducible factor-dependent mechanism [78]. EPO [79] was shown to increase transcription of cyclins, inhibit cell-cycle inhibitors and to increase the concentration of the anti-apoptotic protein, BCLXL [76, 80], favouring red cell proliferation and promoting the differentiation of bone-marrow stem cells into circulating mature red cells. It is produced by renal interstitial cells in the peritubular capillary beds of adult kidney, and by liver perivenous hepatocytes [81–83]. In addition to renal and liver tissues, EPO is also expressed in many extra-renal tissues in cell types such as astrocytes [84], neurons [85] and bone marrow macrophages [86]. It functions as a stimulating factor by binding to a specific erythropoietin receptor (EPOR), a member of the cytokine type 2 family [87–89], which is a 66 kD glycoprotein encoded by a gene on chromosome 19. The EPOR protein consists of a single transmembrane domain and an extracellular domain [90, 91].

The EPOR is widely expressed in non-erythroid cells and tissues such as smooth muscle cells [92], myoblasts [93], vascular endothelium [94], heart [95], kidney [96], neuronal cells [97, 98] and brain [99]. EPOR dimerizes upon EPO binding, which increases the receptor's affinity for JAK2 in the membrane proximal region of the receptor. This results in JAK2/STAT5 protein phosphorylation and activates other signal transduction pathways [91] including the PI3K and extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathways [100–102]. Activation of these pathways prevents apoptosis of late erythroid progenitors by promoting their survival, proliferation and differentiation [102]. In human beings, EPO also enhances megakaryocyte maturation by augmenting platelet counts by 10–20% and increasing platelet function [103, 104].

EPO administration is associated with proliferation of erythroid progenitor cells, which, once differentiated into normoblasts, enucleate and leave the bone marrow [17]. Recombinant EPO is widely used in clinical practice for the treatment of anaemia in patients with end-stage renal failure and patients with anaemia induced by chemotherapy or radiotherapy [105]. A large number of EPO structural and functional variants are now available and under development. The development of a neuroprotective variant with absent or reduced haematopoietic functions represents an important challenge [106, 107].

EPO and experimental stroke

EPO and EPOR are constitutively expressed in the central nervous system and play a significant role during brain development [108]. In neurons, EPO and its receptor activate multiple signal transduction pathways including STAT, PI3K/Akt, Ras/ERK1/2, nuclear factor-kB (NF-kB) and intracellular calcium levels [89]. EPO is neuroprotective in cell cultures. This results in a protective and anti-apoptotic effect on hippocampal neurons [109, 110] as well as on cortical neurons [111, 112] in different *in vitro* studies. Moreover, data from experimental studies provide evidence that EPO and its receptor are overexpressed in response to hypoxia and ischemic damage and have a neuroprotective effect *in vitro* as well as in animal models and human beings [110–113]. EPOR up-regulation occurs first in neurons and endothelial cells in microcirculation after 12 hrs, and is later observed in both neurons and astrocytes. This phenomenon is prominently observed in the penumbra area [85].

The evidence that endogenous EPO inhibition results in more severe brain damage [89] and that EPOR-deficient mice shows increased sensitivity to hypoxia and increased brain apoptosis [91] further support EPO's major role in the brain's response to ischemia. Hypoxia-induced EPO expression is mediated by hypoxia inducible factor (HIF), which may be involved in ischemic tolerance [114].

The anti-apoptotic effect of EPO was shown in different animal models. Studies from neonatal rat and mouse stroke models in showed reduced apoptosis through the differential regulation and expression of genes involved in the apoptotic process [115–118].

A similar anti-apoptotic effect was observed in a gerbil model of cerebral ischemia [119, 120]. Villa *et al.* [120] reported a significant reduction in astrocytes, leucocytes and microglia activation as well as decreased production of pro-inflammatory cytokines like TNF-α, IL-6 and monocyte chemoattractant protein 1 in the ischemic area of a MCAO rat model. The same authors reported a similar anti-inflammatory effect consisting of reduced cellular infiltration, apoptosis and white matter damage after treatment with a non-erythropoietic EPO derivate (CEPO) in a rat model of focal cerebral ischemia [121]. EPO administration was also shown to be associated with improved outcome in an experimental model of traumatic brain injury and with reduced brain oedema [122]. A recent paper showed that EPO reduces neurological symptoms in a mouse model of primary brain oedema [123] supporting the evidence that an important component of EPO's neuroprotective function may be its role in modulating astrocyte water permeability through the regulation of intracellular calcium oscillations, reducing the risk of astrocyte swelling during stroke and other brain insults.

In addition to its anti-apoptotic and anti-oedema effects, EPO exerted pleiotropic effects as an antioxidant [124, 125], anti-inflammatory [120–126], glutamate-inhibitory [127], neurotrophic [128] and angiogenic [88] factors, and influenced stem cell differentiation [88, 91, 129–131].

A recently published meta-analysis including 16 studies (more than 340 animals) with comparable outcome measures assessing the efficacy of EPO administration after experimental focal ischemia reported a significant neurobehavioural improvement (38% limb function, 37% neuroscore and 37% memory function) and a significant infarct size reduction of 32%. In most of included studies (12), treatment was initiated after ischemia onset. In three studies, EPO was administrated before ischemia and in one study

it was evaluated as both a pre- and post-ischemic treatment. A meta-regression of data from six studies supports the evidence that early treatment (within 6 hrs) is most effective. A higher dose also seems to be associated with better outcome. Different EPO derivatives did not differ regarding infarct size reduction and functional improvement [132].

Interestingly, it has been described that the receptors that mediate the neuroprotective effect after ischaemic and traumatic brain injury [133, 134] are different from those expressed by erthroid precursors, [133] and also several EPO variants that do not bind to myeloid cells have a neuroprotective effect [135, 136]. The use of non-haematopoietic EPO analogues could be a promising option in achieving a more specific action and for avoiding systemic side effects such as an increased haematocrit and increased blood pressure values. However, treatment with deferoxamine (DFO), EPO or the combination of DFO and EPO did not protect against grey or white matter damage in the experimental settings used by van der Kooij *et al.* [118]. Kumral *et al.* demonstrated a protective effect for EPO on lipopolysaccharide-induced white matter injury in the developing brain [137].

EPO and human stroke

To our knowledge, there are only two published studies evaluating the safety and efficacy of EPO administered to stroke patients. The first trial by Ehrenreich *et al.* [138] involved 53 patients (13 patients enrolled in a safety study and 40 patients enrolled in a double-blind proof-of-concept trial) with hyperacute ischemic stroke (drug administration within 8 hrs of symptom onset). All patients recruited had an infarct in the MCA area documented by DWI and fluid attenuation inversion recovery MRI in the acute phase. Intravenous infusion of recombinant human (rh)EPO (3.3×10^4 IU/50 ml/30 min.) was started immediately after recruitment and then administered again 24 and 48 hrs later to provide a cumulative dose of 100,000 IU of rhEPO for each stroke patient. MRI data were obtained on days 1, 3 and 18 for each patient. The primary end-points were 30 days NIHSS and Scandinavian Stroke Scale score, and functional outcome on day 30 (Barthel's index and modified Rankin scale). Secondary endpoints were time-dependent fluctuations in neurologic scores and serum markers of injury (S-100B protein) over 30 days, as well as infarct size on days 3 and 18 as assessed by cerebral MRI. EPO was well tolerated and safe in these stroke patients. Moreover, a possible neuroprotective role was suggested by improved clinical outcome (above all, lower Barthel Index score in the treatment arm) and reduced infarct size as well as serum levels of the circulating glial damage marker, S100B in the treatment arm [138].

On the basis of these promising results, a randomized, double-blind, placebo-controlled multicenter study, the German Multicenter EPO Stroke Trial (www.clinicaltrials.gov, NCT00604630), with an accrual goal of over 500 patients, was started in 2003 and concluded in 2008, and confirm the results of the first trial [139].

Ongoing studies

As cited above, there is an ongoing phase II clinical trial of G-CSF and EPO in stroke and amyotrophic lateral sclerosis patients (www.clinicaltrials.gov, NCT00298597). Two other studies evaluating EPO in combination with human chorionic gonadotropin (hCG) are ongoing. The first is the just begun REGENESIS (US) study, a phase IIB study of hCG and EPO in acute ischemic stroke patients, with the primary objective of assessing the safety and tolerability of hCG and EPO in this patient population (start date, August 2009; www.clinicaltrials.gov, NCT00715364). The second is the active but no longer recruiting REGENESIS (CA), a phase II study to assess neurological outcome in acute ischemic stroke patients treated with hCG and EPO (www.clinicaltrials.gov, NCT00663416).

Current evidence and future perspectives

The neuroprotective effect of EPO was demonstrated *in vitro* as well as in experimental models of stroke. Data from two small studies in stroke patients suggest that this hormone is safe and that EPO may have a positive effect on both infarct size reduction and clinical outcome. The development of EPO variants that do not have erythropoietic activity represent a further promising and challenging options for stroke treatment.

Granulocyte-macrophage colony-stimulating factor

Human GM-CSF is a 22 kD glycoprotein encoded by a single gene located on chromosome 5q21–5q32 near the IL-3, IL-4, IL-5, IL-9 and MCSF-R genes [140]. It is a 127 amino acid protein, a member of the haematopoietin receptor superfamily, consisting of cytokine-specific α and common β chains [141]. Other than being responsible for the *in vitro* stimulation of myeloid precursor clonal proliferation [142], GM-CSF was shown to be involved in neutrophils, monocytes, dendritic cell and in macrophage survival and differentiation [143]. GM-CSF is produced by different cell types such as T and B lymphocytes, macrophages, keratinocytes, eosinophils, neutrophils and endothelial cells [144]. GM-CSF function is mediated by activation of the GM-CSF receptor (GMCSFR) which is a cytokine belonging to the type I receptor group. The α-chain of GMCSFR is specific for binding GM-CSF, whereas the β subunit is involved in signal transduction [144]. GM-CSF binding to its receptor regulates cell proliferation through activation of the JAK2/STAT5 and MAPK pathways, but also regulates apoptosis and cell survival through activation of the JAK/STAT and PI3K/Akt pathways [145]. GMCSFR is expressed in $CD34^+$ hematopoietic progenitors, neutrophils, eosinophils, basophils, monocytes, macrophages, microglia, lymphocytes, endothelial cells and mesenchymal cells. It is also expressed in malignancies such us myeloid leukaemia, acute and chronic melanoma, and prostate and lung cancer [146, 147].

The recombinant form of GM-CSF is recognized as a treatment for chemotherapy-induced neutropenia and also employed to enhance the antiviral effects of azidothymidine in HIV patients [148]. Trials have also been conducted to assess GM-CSF use in sepsis, drug-induced neutropenia, HIV infection, acute myeloid leukaemia, aplastic anaemia, myelodysplasia [148] and recently as adjuvant therapy for indolent B-cell lymphoma [149], cutaneous melanoma [150] and chronic lymphocytic leukaemia [151]. The most common dose-related adverse effects are bone pain, erythroderma, weight gain, oedema and inflammation [152].

Increased levels of the pro-inflammatory cytokines IL-8 and GM-CSF were found in the cerebrospinal fluid (CSF) of patients on day 2 after stroke [153], supporting the evidence of a focal immune response in ischemic brain [154].

GM-CSF and experimental stroke

The GMCSFR is broadly expressed in brain regions with a predominantly neuronal pattern in neurons of DG and SVZ [155]. To our knowledge, there are no published studies investigating the neuroprotective effect of endogenous GM-CSF in GM-CSF deficient mouse models. RT-PCR studies reveal that it is expressed in cultured adult stem cells as well as in rodent brain, and that it can enhance neuronal differentiation in adult stem cells in a dose-dependent manner [156].

GMCSFR α gene expression is induced by brain ischemia [155]. Nakagawa *et al.* [157] investigated the therapeutic effect of intracarotid injection of GM-CSF in a rat model of 1-hr intraluminal MCAO. In the treatment group, a significant reduction in infarct size (from 412 mm^3 to 214 mm^3, $P < 0.005$) and a better 48-hr neurological outcome evaluated through Menzie's neurological scale were observed. A significant infarct size reduction was also reported in a mouse model of common carotid artery occlusion (CCAO) [158]. Recently, Kong *et al.* [159] confirmed the neuroprotective effect of intraperitoneally administered GM-CSF in a rat model of MCAO. In the treatment group, a significant infarct size reduction and a favourable clinical outcome (rotarod motor test and motor/sensory) were observed. In addition, GM-CSF administration was shown to modulate the expression of apoptosis-related genes (Bcl-2, Bax, caspase 3 and p53) and to reduce programmed cell death.

Data from the study of Nakagawa *et al.* [157] substantially confirmed the anti-apoptotic function of GM-CSF demonstrated by a reduction in $TUNEL^+$ (apoptotic) cells and by a significant increase in microglia/macrophage ($OX\text{-}42^+$ cells) activation in penumbra area. In culture, GM-CSF administration results in JAK2 kinase pathway activation, PI3K/Akt activation, and in a transient activation of Erk 1/2 that finally results in a substantial anti-apoptotic effect in cortical neurons [159]. In primary cortical neurons and human neuroblastoma cells, GM-CSF counteracted apoptosis and induced Bcl-2 and BCLXL in a dose- and time-dependent manner. Inhibition of the PI3K-Akt pathway resulted in a severe reduction in anti-apoptotic activity [155]. The substantial neuroprotective and anti-apoptotic properties of GM-CSF were demonstrated *in vitro* as well as in *in vivo* experimental stroke models in which administration of GM-CSF led to a decrease in the extent of neuronal apoptosis by modulating the expression of several apoptosis-related genes such as Bcl-2, Bax, caspase 3 and p53 [159]. After neonatal hypoxia in rats, GM-CSF treatment decreased apoptotic cells ($TUNEL^+$ cells), reduced expression of Bax, cleaved caspase-3 and increased the expression of STAT3, Bcl-2 and Pim-1, supporting a neuroprotective effect of GM-CSF administration [160].

Buschmann *et al.* [161] first demonstrated an improvement in brain hemodynamic parameters after GM-CSF treatment in a rat model of cerebral ischemia due to the occlusion of the vertebral plus the left carotid artery [3-VO]. GM-CSF induced a posterior cerebral artery diameter enlargement and enhanced mononuclear cell invasion (macrophages) at the site of vascular collateral proliferation. In other studies in the same experimental model, GM-CSF was shown to protect against hypotension-induced cerebral blood flow (CBF) decline and hypotension-induced ATP depletion, resulting in a reduction in metabolic injury [162].

In a rat model of sequential bilateral carotid artery occlusion, GM-CSF treatment induced restoration of impaired cerebral hemodynamic and favoured structural changes in the resistance-vessel network. In the treatment arm, there was a significantly higher number of intraparenchymal vessels per section than in control groups as well as a higher density of leptomeningeal vessels per section. Moreover, one week after artery occlusion, $CD\text{-}68^+$ cell numbers were higher in the perivascular area of GM-CSF treated animals [163]. The more recent report from Todo *et al.* [158] confirmed the role of GM-CSF in enhancing leptomeningeal collateral growth in a mouse model of unilateral CCA occlusion. In the same study, a significant infarct size reduction in the GM-CSF group was detected.

GM-CSF and human stroke

Despite experimental evidence for the role of GM-CSF in inducing vascular remodelling, restoration of cerebral hemodynamic and mobilization of $CD34^+$ cells [164, 165] after cerebral ischemia, no clinical trials have been implemented or are ongoing to date. A small, recently published study in patients with acute stroke showed that stroke is associated with higher plasma levels of GM-CSF but failed to find an association between GM-CSF levels and better neurological outcome. This finding supports a role for GM-CSF in response to brain ischemia, but further studies are necessary to better clarify its function [166].

Current evidence and future perspectives

In vitro, GM-CSF enhances neuronal differentiation and prevents apoptosis. A substantial anti-apoptotic effect was confirmed in animal models in which GM-CSF administration resulted in improved functional outcome and brain hemodynamic, and infarct

size reduction. Despite these promising results and that GM-CSF is already licensed for various clinical uses, studies in stroke patients have not yet been implemented. For its role in remodelling the vascular architecture, GM-CSF may be a suitable candidate for future stroke therapy.

Vascular endothelial growth factor

VEGF or VEGF-A is a 23 kD glycoprotein belonging to a family of heparin-binding GFs including VEGF-B, VEGF-C, VEGF-D and placental-like GF. VEGF is encoded by a gene on chromosome 6 and can be alternatively spliced into at least five different isoforms in human beings (VEGF121, VEGF145, VEGF165, VEGF189 and VEGF206). The mouse VEGF-A isoform has one less amino acid [167] and is responsible for endothelial cell proliferation, migration and survival [168]. First discovered for its regulatory function in vascular endothelial cell permeability, it is now a well-known angiogenic factor with a recognized role in vasculogenesis and vascular maintenance in all mammalian organs.

VEGF is a multifunctional protein involved in capillary regulation in adult skeletal muscle, angiogenesis, inflammation, cancer and wound healing and repair. It is also expressed in epithelial cells in the kidney and lung [167]. VEGF is ubiquitously expressed in brain, mostly by choroid plexus epithelial cells but also by astrocytes and neurons [169, 170].

Many different factors, including both genes and gene products, are known to enhance VEGF production. VEGF is secreted by tumours in response to hypoxia to stimulate angiogenesis to increase oxygen supply [171], but its production is also triggered by oncogenes (c-Src, Bcr-Abl and Ras oncogenes), tumour suppressor genes (*e.g.* p53) [172–175] and other GFs and cytokines such as cyclooxygenase-2 [176] and platelet derived growth factor (PDGF) [177].

VEGF's biological functions are mediated by its binding to receptors on vascular and lymph-vessel endothelial cells as well as on other cell types including fibroblasts, smooth muscle cells, haematopoietic stem cells, epithelial cells, monocytes and macrophages [178]. Three VEGF receptors, VEGFR-1 (flt-1), VEGFR-2 (KDR/flk-1) and VEGFR-3 (flt-4) with tyrosine kinase activity have been identified. Co-receptors, including heparin sulphate proteoglycans and neuropilins, may modify the tyrosine kinase activity of VEGFR or bind VEGF directly.

VEGFR-1, which binds VEGF-A, B and PLGF, regulates vascular development and signal transduction mediated through ligand-specific patterns of receptor phosphorylation [179]. VEGFR-2, which binds VEGF-A, is important for angiogenesis; VEGFR-3, which binds VEGF-C and VEGF-D, influences lymphangiogenesis.

Neuropilin co-receptors (Np-1 and Np-2) influence vascular endothelial and non-endothelial cells (*i.e.* podocytes), the protection of neuronal cells, axon growth and guidance, and modulate VEGF distribution in various tissues [180].

VEGF is actually considered one of the most important angiogenesis regulators and a new key target in anti-cancer treatment. The role of anti-VEGF or anti-VEGFR therapy is supported by clinical trials. Currently, the humanized monoclonal antibody, bevacizumab, which binds to VEGF-A, and the small molecule kinase inhibitors sunitinib and sorafenib (which inhibit the kinase activities of VEGFR) were approved for use in patients with various malignancies.

VEGF and experimental stroke

In vitro, VEGF protects cultured motor neurons from hypoxia, reactive oxygen species, and glutamate-induced toxicity [181, 182] and acts as a trophic factor for neuronal stem cells [183]. Moreover, VEGF and VEGFR expression were induced in brain after global ischemia [184] or permanent [185] or transient MCAO, with a different temporal profile among these cells [186–188].

In hypoxic and ischemic brain, VEGF expression is induced by transcriptional activation *via* HIF-1 and HIF-2 [170, 185, 189, 190]. Within 6 hrs after ischemic injury, VEGF expression was increased in the penumbra area [185]. VEGF, VEGFR2 and HIF-1, through their angiogenic functions, seem to be protective in long-term responses to hypoxic injury, even if, in the acute phase, their expression favours oedema formation [191].

VEGF-A was also expressed during hypoxic preconditioning in adult mouse [192]. Immunohistochemistry of brain tissue from adult rats subjected to photothrombotic ring stroke with spontaneous reperfusion showed that the VEGF protein subtypes A and C increased simultaneously 2 hrs after irradiation of the lesion, and from 24 to 72 hrs after stroke in the penumbra-like zone. The expression of VEGF A, C, and their respective receptors (flt-1, flk-1 and flt-4) was also associated with microvessel formation [193]. Since VEGF plays a positive role in angiogenesis, remodelling and re-vascularization of damaged tissues [191] GF has recently been under investigation in ischemic brain [194].

eNOS expression after brain ischemia following permanent MCAO was found to temporarily and anatomically co-localize with VEGF expression mostly in the penumbra area where these proteins probably exert a protective effect on damaged brain tissue [195]. Furthermore, in a rat model of focal embolic cerebral ischemia, exogenous nitric oxide administration was shown to enhance angiogenesis and vasculogenesis by activating the nitric oxide/cyclic guanosine monophosphate pathway and by increasing VEGF levels in ischemic boundary regions [196]. Mice overexpressing the human VEGF165 isoform exhibited a physiologically higher density of brain vessels and enhanced angiogenesis after brain ischemia. Mice overexpressing VEGF also had a smaller infarct area, which correlated with better clinical outcome [197, 198].

VEGF overexpression was associated with a higher capillary density, but not with an increase in blood flow, probably because circulating blood is stolen by the non-ischemic areas [197]. Another experimental model in which recombinant human VEGF165 was infused into the right lateral ventricle of rats 1 week

before MCAO showed that VEGF was able to decrease infarct volume and brain oedema without increasing CBF [199].

Several experimental studies substantially confirmed the positive effect of VEGF in ischemic stroke. Transient MCAO is the most common model despite a great deal of variability between different protocols in terms of the administration route (see Table 1).

VEGF administration, as previously reported for other GFs, was associated with an infarct size reduction ranging from 34% to 47% [199–202] and improved clinical outcome [198, 202–205] without an increase in brain oedema [199, 202]. The relationship between VEGF and brain oedema has been investigated by studies aimed at clarifying the possible underlying mechanisms of brain oedema. The evaluation of VEGF and angiopoietin (Ang-1 and -2) expression in a rat model of irreversible MCAO by Beck *et al.* [206], showed that, in the early phase after ischemic damage, Ang-2, which plays a role in vascular remodelling by blocking the constitutive expression of Ang-1, is hypoxia induced. Thereafter, Ang-2 expression is likely to follow VEGF up-regulation, since VEGF protein was first detected after 6 hrs with expression peaking after 24 hrs [206]. The combined effects of VEGF and Ang-2 on cerebral angiogenesis was confirmed by Zhu and colleagues, who also reported that Ang-2 and VEGF act in combination in BBB disruption [207]. This effect on the BBB was previously reported in a rat model of focal cerebral ischemia in which MRI) showed that post-ischemic (1 hr) administration of $rhVEGF_{165}$ significantly increases BBB leakage, haemorrhagic transformation and ischemic lesions, whereas VEGF administration after the acute phase (48 hrs) was associated with angiogenesis enhancement and functional improvement [203].

The link between VEGF and BBB after stroke was further investigated by Zhang *et al.* in a rat model of embolic stroke where VEGF mRNA was found to be increased 2 to 4 hrs after stroke, whereas Ang-1 mRNA decreased [208]. There was an association between VEGF up-regulation and BBB leakage in the ischemic core. VEGF/VEGFRs and Ang/Tie2 were detected at the boundary of the ischemic lesion from two to 28 days after stroke in association with newly formed vessels. These data support a possible role of Ang-1 in limiting VEGF-induced BBB leakage during the acute phase, whereas up-regulation of VEGF/VEGFR and Ang/Tie2 at the boundary zone may regulate neovascularization and angiogenesis in ischemic brain tissue [206–208]. Zhang and Chopp administered exogenous VEGF in a mouse model of focal cerebral ischemia showing that increased levels of Ang-1 reduces BBB leakage [209]. Mesenchymal stem cell (MSC) infusion 24 hrs after MCAO significantly decreased BBB leakage and promoted angiogenesis by increasing endogenous Ang1 and Tie2, and occluding cerebral expression in the ischemic border. Capillary formation was reduced after inhibition of Flk1 or Ang-1 and down-regulation of Tie-2, suggesting a critical role for VEGF/Flk1 and Ang1/Tie2 in MSC-induced angiogenesis [210].

In a mouse model of brain ischemia, inhibition of VEGF activity using a neutralizing antibody was shown to completely block hypoxia-induced vascular permeability [211]. A similar effect was obtained by employing the VEGF antagonist in a rat model of vasogenic oedema due to cerebral venous ischemia [212]. In a 90-min. MCAO rat model, pre-treatment with a KDR kinase inhibitor elicited an early, transient decrease in oedema and subsequent reduction in infarct volume, implicating VEGF as a mediator of stroke-related vascular permeability and ischemic injury [201].

The role of VEGF in stroke-oedema is confirmed by further experimental models. Stereotactic injection of VEGF into the left substantia nigra (SN) resulted in highly up-regulated aquaporin-4 (AQP4), which is the most abundant water channel in the brain. AQP4 mRNA and protein in the ventral midbrain localized in close proximity with the new VEGF-induced blood vessels suggest a positive role of AQP4 in oedema resolution, which may partially explain the previously reported beneficial effects of delayed VEGF administration in ischemic rats [213].

In a gerbil model of focal brain ischemia, pre-treatment (6 days or 12 days) with an intracerebroventricular injection of adeno-associated virus transferring the gene for VEGF (rAAV-VEGF) improved survival and brain oedema, and delayed neuronal death [214]. However, this method of administration is controversial with some authors reporting that intraventricular rAAV-VEGF pre-treatment in the transient MCAO rat model can result in deleterious intracranial hypertension and augment secondary ischemic insults during the early stages. They concluded that pre-ischemic VEGF gene transfer *via* an intraventricular approach may not be a favourable therapeutic strategy for MCAO, which should be adopted with caution or avoided in experimental stroke [215].

Also, different dosages of VEGF165 can injure rather than promote recovery of nervous tissue [216]. In this experimental model, VEGF165 was administered intra-arterially at different dosages in temporary MCAO rats. In this study, brain treated with low non-angiogenic doses of VEGF165 showed preserved neuropils and reduced numbers of macrophages in comparison with ischemic brains treated with high angiogenic doses, which showed phagocytized neuropils and a high macrophage density.

Both HIF-1α and VEGFR-2 were implicated in peri-infarct neuroprotection. In squirrel monkeys, a bipolar electro-coagulation of cortical vessels supplying the M1 hand area representation was performed. It was shown that HIF-1a up-regulation was confined to the infarct and peri-infarct regions. Increases in VEGFR-2 immunoreactivity occurred in two remote regions. Thus, while remote areas undergo a molecular response to the infarct, the authors suggested that there is a delay in the initiation of the response, which may ultimately increase the 'window of opportunity' for neuroprotective interventions in the intact cortex [217].

Regarding new insights in VEGF mediated angiogenesis, MCAO was performed in mice overexpressing (VEGF-hi/+) and underexpressing (VEGF-lo/+) VEGF. Compared with VEGF-hi/+ mice, VEGF-lo/+ mice formed fewer collaterals during the perinatal period when adult density was established, and had a 2-fold larger infarction after MCA ligation, suggesting that VEGF and VEGFR-1 are determinants of arteriogenesis. VEGF-A specifies formation of native collaterals in healthy tissues [218].

In cerebral ischemia, Chu *et al.* investigated the effects of combined intravenous administration of transplanted neural stem cells and VEGF on focal cerebral ischemia in rats. Combined cell and pharmacological therapy was associated with better functional recovery (improved sensorimotor deficit and better performance on

the behaviour tests), reduced cerebral atrophy and increased vascular density without a significant effect on stem cell survival [205].

After transplantation of neural progenitor cells (NPCs) expressing VEGF-A165 into the caudate putamen in rats, Maurer *et al.* [219] found an increased survival of adenovirally transfected NPCs and an increased expression of the endothelial cell marker, PECAM-1 (CD31) demonstrating that the graft itself is a useful vehicle for GF delivery, promoting the survival of NPCs. Moreover, transplantation of VEGF-expressing NPCs supports angiogenesis in the brain, which may contribute to brain repair.

Intracerebral administration of VEGF gene-transferred BMSCs engineered with a replication-deficient herpes simplex virus type 1 1764/4-/pR19-hVEGF165 vector in a rat model of MCAO determined a more significant functional recovery, decreased infarct volume and showed stronger expression of VEGF proteins compared with transplantation of native BMSCs alone [220]. Moreover, the neuroprotective functions of VEGF were also mediated by its anti-apoptotic role as shown by a significant reduction in TUNEL staining in the VEGF-treated group, both at 24 and 48 hrs in the study of Hayashi *et al.* [200].

VEGF and human stroke

No clinical studies with VEGF have been implemented so far in ischemic stroke patients.

Ongoing studies

One study is active but not ongoing. It focuses on angiogenesis after intracardiac injection of VEGF (phase I) and oral L-arginine supplementation (phase II) in coronary artery disease patients. This study could provide useful information on the effects of VEGF on angiogenesis after ischemic injury (www.clinicaltrial.gov, NCT00134433).

Current evidence and future perspectives

VEGF is a trophic factor for neuronal stem cells and its expression increases after cerebral ischemia. In animal models, VEGF administration results in better functional outcome and reduced infarct size and oedema. Currently there are no data from studies assessing the safety and efficacy of VEGF in stroke patients.

Stromal cell-derived factor-1

SDF-1 is a CXC chemokine produced by bone marrow stromal cells. Two isoforms, SDF-1α and SDF-1β, arising from alternative splicing of the SDF-1 mRNA, are identified. The resulting proteins, SDF-1α and SDF-1β are identical except for four C-terminal amino acids and represent endogenous ligands for the CXC chemokine receptor 4 (CXCR4), which also functions as co-receptor for HIV-1 in lymphocytes [221–223]. SDF-1 is a potent chemoattractant for hematopoietic stem cells (HSCs) and $CD34^+$ cells, which express CXCR4. Thus, SDF-1α, which is constitutively expressed in all tissues, plays an important role in HSCs trafficking between the peripheral circulation and bone marrow [224, 225]. In brain, SDF-1/CXCR4 is recognized as being expressed in a variety of neuronal tissues during development, including cerebellum [226], cerebral cortex [227, 228], DG [229] and motor axons [230], where it was shown to regulate synaptic transmission and neural cell migration [231, 232] and to guide migration of neural progenitors from the external germinal layer to the internal granular layer [233–235]. The detection of increased SDF-1α expression in the brain of HIV-positive patients supports the notion that SDF-1α may also play a role in neuroprotection and neuronal plasticity [236]. SDF-1 up-regulates the expression of VEGF in neurovascular endothelial cells [237]. In addition, the SDF-1α/CXCR4 axis is related to arginine vasopressin release in adult brain [238–240] and microglia-enhanced, SDF-1-stimulated, TNF-β and glutamate release from astrocytes *in vitro*. This mechanism is also proposed as a novel excitotoxic pathological mechanism in some neurodegenerative diseases [241].

Expression of both SDF-1α and CXCR4 was up-regulated, mostly under hypoxic conditions such as acute renal failure, ischemic cardiomyopathy and ischemic brain injury [242–244].

Liu *et al.* reported that synchronized Ca^{2+} spikes among cultured hippocampal neurons, which represent periodic burst firing of action potentials believed to play a major role in the development and plasticity of the neuronal circuitry, can be modulated by two small factors that act on G-protein-coupled receptors (GPCRs): the neuropeptide pituitary adenylate cyclase-activating polypeptide, and the chemokine SDF-1. This modulation of neuronal activity through GPCRs may represent a significant mechanism that underlies the neuronal plasticity during neural development and functioning [245].

Stumm *et al.* (2002) demonstrated that an isoform-specific regulation of SDF-1, obtained by alternative splicing, modulated neurotransmission and cerebral infiltration through distinct CXCR4 pathways. After focal ischemia, SDF-1β expression increased in the endothelial cells of the penumbra blood vessels and decreased in non-lesioned brain areas, whereas there was concomitant infiltration of CXCR4-expressing peripheral blood cells. In the same experimental model, neuronal SDF-1α was transiently down-regulated and neuronal CXCR4 was transiently up-regulated in the non-lesioned cortex after ischemia. Thus, whereas SDF-1β may control cerebral blood cell infiltration, the neuronal SDF1-α/CXCR4 system may contribute to ischemia-induced neuronal plasticity, suggesting that the isoform-specific regulation of SDF-1 expression modulates neurotransmission and cerebral infiltration [246].

SDF-1 in experimental stroke

Stumm *et al.* [246] showed that SDF-1 is up-regulated in peri-infarct and infarct tissue in a mouse stroke model, and that this

up-regulation was accompanied spatially and temporally by CXCR4-expressing infiltrates.

The results of this study were confirmed in other experimental models. Hill *et al.*, using anti-SDF-1 antibodies, detected increased expression of SDF-1 protein in MCAO stroke rats [247]. In this study, SDF-1 expression was observed in infarcted hemispheres within 24 hrs, increased by 7 days after MCAO, and was maintained for at least 30 days after occlusion of the MCA. SDF-1 expression was principally localized to ischemic penumbra, and appeared to increase as one moved from the periphery of the penumbra in towards the border of the infarct core. The prominent expression of SDF-1 in the penumbra area when new blood vessels are forming supports the role of SDF-1 in brain remodelling after ischemic injury. The authors also reported an association between transplanted bone marrow derived cell location and density and the level of SDF-1 immunoreactivity on day 14 after occlusion. These findings suggest that SDF-1 has an important role in bone marrow-derived cell homing, especially monocytes, to areas of ischemic injury.

The extended expression of SDF-1 in the penumbra, and at a later time in the ischemic core when new blood vessels are forming, is concordant with its being involved in some aspects of brain remodelling following ischemic injury.

Thus, there is considerable evidence for SDF-1s having a role in targeting neuronal precursors as well as neural and bone marrow-derived MSC towards an ischemic brain lesion [242, 248–253]. CXCR4 was identified in tissue-committed bone marrow stem cells, which express neural lineage markers and may become attracted to ischemic brain tissue *via* SDF-1 [254].

The role of this chemokine as a signal adhesion molecule and in inducing migration of HSCs to injured brain tissue in an ischemic animal model was further demonstrated by Shyu *et al.* [255]. These authors injected recombinant SDF1α protein intracerebrally into MCAO rats and treated cells in culture with SDF-1. This treatment increased engraftment of BrdU-labelled bone marrow derived cells in the ipsilateral cortex near the ischemic area boundary and in the sub-ventricular region of the ischemic hemisphere.

The same authors performed an intracerebral injection of human olfactory ensheathing cells (OECs)/olfactory nerve fibroblasts (hOECs/ONFs) in a stroke model. Transplanted and endogenous homing stem cells co localized with specific neural and vascular markers, suggesting a stem-cell fusion phenomena. Interestingly, both hOECs/ONFs and endogenous homing stem cells enhanced neuroplasticity in rat and mouse ischemic brain models by up-regulation of SDF-1α and CXCR4 [256].

A great increase in SDF-1 and CXCR4 expression in the infarct and peri-infarct regions 2–10 days after stroke was also recently reported by Schonemeier *et al.* [257] in MCAO rats. These results support the possible role of a SDF-1 a/CXCR4 interaction in adaptive reorganization and inflammation of post-ischemic injury other than in vascular remodelling, angiogenesis and neurogenesis, thereby alleviating stroke symptoms. Since the infarct volume was significantly reduced in SDF-1α treated rats compared with controls 3 days after stroke, the authors suggested that SDF-1α may provide a neuroprotective function against neurotoxic insult and exert an anti-apoptotic effect [257]. To our knowledge, SDF-1 has never been tested in stroke patients.

Current evidence and future perspectives

SDF-1 is up-regulated in different hypoxic conditions and acts mostly as a signal adhesion molecule in inducing and targeting cell migration and homing towards ischemic lesions. To our knowledge, SDF-1 has never been tested in stroke patients. However, improvement in locomotor activity tests and the reduction of infarct volume 3 days after stroke observed in rats treated with SDF-1 could support the use of SDF-1 in inducing brain plasticity and functional recovery.

Stem cell factor

SCF is an essential haematopoietic cytokine that interacts with other cytokines to preserve the viability of hematopoietic stem and progenitor cells, influencing their entry into the cell cycle and facilitating their proliferation and differentiation. When combined with other cytokines, SCF increases the cloning efficacy of hematopoietic progenitor cells from all lineages and the clonogenic activity of other CSFs [258].

SCF alone cannot drive non-cycling haematopoietic progenitor cells into the cell cycle, but can prevent these cells from undergoing apoptosis. Particularly, SCF and G-CSF favour neurogenesis and have a neuroprotective effect partly mediated by up-regulation of anti-inflammatory cytokines such as IL-10 [61]. Moreover, SCF plus G-CSF augment bone marrow derived cells that express the neuronal marker, NeuN in intact brains [259] as well as in acute and subacute ischemic brains [59]. The combined use of these GFs also supports the mobilization of BMSCs into the brain and their differentiation into neuronal cells. SCF and G-CSF administration, in the acute phase after focal ischemia induced by permanent MCAO, was associated with infarct volume reduction [59]. In a rat model, SCF administered in the acute phase and within the 7 days after experimental stroke was associated with enhanced neuronal progenitor cell proliferation, reduced infarct size and improved clinical outcome. In this study, functional improvement was delayed in SCF-treated animals, but was long lasting compared with G-CSF-treated animals [260]. The same authors demonstrated a beneficial effect of SCF treatment in an animal model of chronic brain ischemia [260, 261] showing that systemic administration of SCF plus G-CSF during the chronic phase of brain ischemia, initiated 3.5 months after ischemia for 7 days, led to improved functional recovery with a reduced size of the infarct cavities. However, the mechanisms by which SCF plus G-CSF help to repair the damaged brain during chronic stroke remains unknown. Administration in the subacute phase similarly reduced infarct area, but also promoted the generation of neuronal cells from both bone marrow derived cells and intrinsic neural stem/progenitor cells, resulting in improved functional recovery [261].

Current evidence and future perspectives

This observation indicates that combined SCF and G-CSF treatment may favour mobilization of BMSCs into the brain and their differentiation into neuronal cells in both acute and chronic stroke, leading to improvement of functional recovery with reduced infarct size. No studies on the use of SCF alone are available. Despite the absence of available clinical trials, the results of experimental studies provide interesting insights into the use of SCF and G-CSF in chronic stroke.

Other growth factors: neurotrophins

It has been observed that cerebral ischemia up-regulates expression of several GFs such as brain–derived neurotrophic factor (BDNF), fibroblast growth factor (FGF)-2 and IGF-1, epidermal growth factor (EGF) and glial cell line-derived neurotrophic factor (GDNF) [262].

Neurotrophins are a family of structurally related polypeptides that play a critical role during neuronal development and appear to mediate a protective response in mature animals [263]. Neurotrophins demonstrated a protective role in several experimental stroke studies (Table 2). Human mesenchymal stem cells (hMSCs) have the capacity to secrete neurotrophins in culture [264–267]. hMSCs respond to the ischemia-injured brain environment through an increased production of neurotrophins and angiogenic factors [268].

Brain–derived neurotrophic factor

BDNF, a member of the nerve growth factor (NGF) family, is the most abundantly expressed neurotrophin in the mature CNS. It acts through a high-affinity cell surface receptor (TRKB) [269], supports the survival of many types of neurons and prevents neuronal death after traumatic events like cerebral ischemia by activating intracellular protein kinases B, MAPKs and ERKs [270]. BDNF is also related to synaptic and axonal plasticity associated with memory, learning and sensory-motor improvement [271, 272]. It also increases the number of neurons in newborns and induces neurogenesis [273–275]. With regard to these findings, BDNF knockout mice developed larger infarcts, and blockade of endogenous BDNF reduced the survival of neurons after an ischemic insult [276, 277]. Intrastriatal infusion of BDNF in adult rats before ischemia induced increased neuronal survival and improved functional recovery [278].

Ferrer *et al.* [279], in an ischemia model of MCAO rats, found a permanent reduction of BDNF and its full-length receptor, TRKB in the infarcted core as well a transient increase in BDNF immunoreactivity in the penumbra area 12 hrs after MCA occlusion. The authors proposed a role for BDNF in preventing neuronal death in the penumbra area by up-regulating the full-length TRKB receptor.

In 1997, Schabitz *et al.* [280] showed that pre-treatment of MCAO rats with intraventricular BDNF reduces infarct size. These results were confirmed by Nomura *et al.* [267], who demonstrated that intravenous administration of hMSCs expressing the BDNF gene in MCAO rats reduced the infarct volume. These favourable effects were proven only in acute phase stroke, whereas later delivery seems to attenuate intrinsic neurogenerative responses [281]. After transient MCAO in $BDNF^{+/-}$ mice were compared with wild-type mice, BDNF was shown to provide functional improvement by inducing neurogenesis and not by influencing infarct volume. To support these results in $BDNF^{+/-}$ mice, the number of neuroblasts in the striatum was found to be significantly increased [282]. However, since BDNF does not seem to cross the BBB, it can be argued as to whether administration of this factor, when the BBB is not disrupted, is protective in brain ischemia [283, 284]. Thus, the most effective means of administration remains to be assessed.

Current evidence and future perspectives

After transient MCAO, BDNF mRNA is up-regulated in cortical neurons suggesting that BDNF potentially plays a neuroprotective role in focal cerebral ischemia. No clinical studies are available using BDNF in stroke patients. Despite promising results from a preclinical study mostly in the acute phase of stroke, further experimental studies should be implemented to assess whether intravenously administered BDNF could be effective in stroke recovery before starting clinical trials.

Basic fibroblast growth factor

bFGF or FGF2 is probably the most potent neurotrophic factor in the FGF superfamily, and has mitogenic, angiogenic and neurotrophic properties [285]. Experimental studies showing that the progenitor cell population in the SVZ is reduced 50% in FGF2 knockout rats suggest that bFGF is involved in neurogenesis [286].

bFGF is widely distributed in neuronal and non-neuronal tissues and has additional effects on mitogenic activity and vasodilation [287, 288]. Preliminary experimental studies showed up-regulated expression of FGF in brain after ischemic injury [289]. Additionally, bFGF is implicated in neurogenesis; after endogenous administration of FGF, cells co localize with neurons and markers of proliferating cells such as BrdU and NeuN [290].

Intravenous administration of high doses of bFGF within hours after stroke onset reduces infarct size (24–50% reduction of infarct volume) in animals subjected to MCAO [291], showing that intravenous bFGF crosses the damaged BBB [292]. These effects were confirmed by Li *et al.* [293], who showed that infusion of bFGF 2 hrs after stroke onset significantly improved sensorimotor functions and reduced infarct volume. Although the mechanism of neuroprotection by intravenous bFGF is unclear, it is supposed that it is related to a direct cytoprotection of the cells in the penumbra area [293].

Table 2 Neurotrophins experimental studies

Neurotrophins	Study reference	Animal model	Sample size (treatment group/control group)	Type of study (focal or global ischemia)	Treatment	Time of administration	Results
IGF-1	[318]	Rat	*N*° Experiments: *n*° animals; 1: 20 mcg IGF-1 (= 18); 1: 20 mcg IGF-2 (= 20); 1: 20 mcg des-IGF-1 (= 18); 2: Vehicle (= 12); 2: 2 mcg des-IGF-1 (= 12); 2: Vehicle (= 26); 2: 150 mcg des-IGF-1 (= 26); 3: Vehicle (= 20); 3: 20 mcg IGF-1 (= 20); 3: 20 mcg IGF-1 + 30 mcg IGF-2 (= 20); 4: [3H]IGF-1/20 mcg IGF-1 (= 4); 4: [3H]IGF-1/20 mcg IGF-1 + 30 mcg IGF-2 (= 4)	Right CCA ligation, and after recovery from anestesia and 10 min. of inhalational hypoxia	I.c.v. infusion of recombinant human IGF-1 and/or IGF-2, at different dosages, or vehicle alone.	2 hrs after hypoxia	20 mcg IGF-1 reduced neuronal loss in all regions. Neither 20 mcg IGF-2, 2 mcg des-IGF-1, nor 20 mcg des-IGF-1 reduced neuronal loss. There was a trend towards a reduction in neuronal loss after 150 mcg des-IGF-1. IGF-2 alone increased neuronal loss in the hippocampus and DG compared with vehicle-treated animals. Coadministration of 30 mcg IGF-2 blocked the neuroprotective effects of 20 mcg IGF-1 and reduced the accumulation of F3HJIGF-1 in the injured hemisphere.
	[320]		Group A, *n* = 13: i.c.v. treated with IGF-I; Group B, = 14: placebo; Group D, = 10: s.b. treated with IGF-I; Group E, = 10: placebo; Groups C and F sham-operated controls (= 5 and = 3, respectively).	Transient right MCAO for 1 hr	I.c.v.: 33.33 mcg IGF-I/d for 3 days, 200 mg IGF-I/d for 7 days	Treatment was begun 30 min. after MCAO	There was less neurological deficit after ischemia in i.c.v. and s.c. IGF-I–treated animals compared with controls. Continuous treatment with i.c.v. and s.c. administered IGF-I achieved a long lasting neuroprotective effect as early as 24 hr after ischemia.
	[322]	Mice	8 for each group	3 weeks after gene transfer the mice underwent permanent distal MCAO.	Long-term cerebral IGF-1 overexpression by the AAV transduction system through stereotaxic injection. Control mice were injected with AAV–green fluorescent protein or saline.		IGF-1 gene transfer compared with control treatment significantly improved motor performance, demonstrated reduced volume of cerebral infarction. IGF-1 gene transfer potently increased neovessel formation in the peri-inf arct and injection needle tract area compared with AAV–GFP transduction. Increased vascular density was associated with increased local vascular perfusion. AAV-IGF-1 treatment enhanced neurogenesis in the SVZ compared with AAV–GFP treatment.

Continued

Table 2 Continued

Neurotrophins	Study reference	Animal model	Sample size (treatment group/control group)	Type of study (focal or global ischemia)	Treatment	Time of administration	Results
	[323]	Diabetic animals		MCAO	i.v. infusion of IGF-1	Acute administration of IGF-1 30-min. before or 2 hr after MCAO followed by 24-hr reperfusion	Significant decrease of both lesion volume and apoptosis
	[317]	Rat	n = 3/time-point/set: -1, 5 and 10 hrs, 1, 3, 5 and 10 days after 60 min. hypoxia -1, 3 and 5 days after the 15-min. hypoxia	Right CA ligation. Hypoxia for 15 or 60 min.			Authors found cell type-specific expression for IGF-1, IGFBP-2, 3, 5 and 6 after injury. IGF-1 produced by microglia after injury is transferred to perineuronal reactive astrocytes expressing IGFBP-2
	[302]	Mice and LID mice		30 min. MCAO	human IGF-I (hIGF-I, 50 μg/kg/day, by a s.c. long-term delivery system	Before MCAO	Authors found that chronic high serum IGF-I correlates with increased brain infarct size following MCAO, while low levels correlate with reduced lesion size, suggesting that lowering serum IGF-I levels in aging mammals, may beneficial against the increased risk of stroke associated to old age.
	[319]	Late gestation fetal sheep	6 per group	30 min. bilateral inflation of carotid artery	0.1 mcg rhIGF-1, 1 mcg hIGF-1, 10 mcg rhIGF-1, or vehicle was infused into a lateral cerebral ventricle over 1 hr.	2 hrs after MCAO	Overall neuronal loss was reduced with 0.1 mcg and 1 mcg rhIGF-1, but treatment with 10 mcg was not effective. With 1 mcg rhIGF-1 neuronal loss scores were significantly lower in cortex, hippocampus, and striatum, 1 mcg rhIGF-1 also delayed the onset of seizures and reduced their incidence.
	[321]	Rat	150 μg rhIGF-I (n = 10); 37.5 μg rhIGF-I (n = 10); controls (n = 9).	Permanent MCAO	Intranasal delivery of vehicle-control, 37.5 and 150 μg of recombinant human IGF-I (rhIGF-I)	At 10 min. after onset of 2 hrs of MCAO, and then 24 and 48 hr later	Treatment with the 150 μg IGF-I significantly reduced the infarct volume *versus* control and improved all the neurologic deficits. The 37.5-μg dose of IGF-I was ineffective.

Continued

Table 2 Continued

Neurotrophins	Study reference	Animal model	Sample size (treatment group/control group)	Type of study (focal or global ischemia)	Treatment	Time of administration	Results
GDNF	[333]		rLV-GFP, $n = 20+5$; rLV-GDNF, $n = 12+4$; non-transduced, $n = 6$; rAAV-GFP, $n = 8$; rAAV-GDNF, $n = 8$	30 min. of MCAO	1° experiment: intrastriatal injections of rLV carrying the gene for GFP (rLV-GFP, or GDNF (rLV-GDNF) or were given an incision of the skin overlying the skull only; 2° experiment: rats were given injections of rAAV carrying the gene for GFP (rAAV-GFP, or GDNF (rAAV-GDNF), into the SN	1° experiment: 4 weeks prior to MCAO or sham surgery; 2° experiment: 6 weeks prior to 30 min. of MCAO	The two routes of GDNF gene delivery both effectively promoted high striatal GDNF levels, which persisted several weeks after the stroke. Contrary to previous experiments, the treatment did not increase neuron survival or improve functional recovery. The authors proposed that the net effect of is complex and may depend on several experimental factors
	[329]	Rat	9+7/7+5+6 controls	MCAO	GDNF (2.5 mcg) or vehicle were placed in contact with the surface of the cerebral cortex.	After MCAO	Both infarct size and brain edema after permanent MCAO were significantly reduced by topical application of GDNF. Induction of TUNEL staining and immunoreactivities for caspases-1 and -3 was greatly reduced with GDNF treatment.
	[330]	Rat	59	40 min. transient middle MCAO and BCCAO	Human recombinant GDNF was injected initially i.c.v. (0.4 mcg/mcl $\times$ 10 mcl). Three injections of GDNF (0,4 mcg/μl $\times$ 5 mcl X 3 sites) were made directly into the cortex adjacent to the MCA.	5 min. after intracerebral GDNF Injection, the left MCA and bilateral CCAs were ligated for 40 or 90 min. in the aged and young animals, respectively.	GDNF protects the cerebral hemispheres from damage induced by MCAO. The increase in nitric oxide that accompanies MCAO and subsequent reperfusion is blocked almost completely by GDNF.

Continued

Table 2 Continued

Neurotrophins	Study reference	Animal model	Sample size (treatment group/control group)	Type of study (focal or global ischemia)	Treatment	Time of administration	Results
	[331]	Rat	Vehicle = 5+4+2; Ad–LacZ = 5+4; Ad–GDNF 6+4+2; 1 hr after Ad–GDNF = 6+4+2; Sham control sections = 2	90 min. of transient MCAO	Ad–LacZ or vehicle solution was administered to the ipsilateral cortex . Ad–GDNF (10 μl).	Just, or 1 hr after reperfusion	Ad–GDNF significantly reduced the infarct volume when immediately administered after the reperfusion, but became insignificant when administered at 1 hr after the reperfusion. The protective effect of GDNF was related to the significant reduction of the number positive cells positive cells for active caspase-3 but not -9.
	[332]	Rat	Rats were killed after 2, 6, 24 hrs, or 1 week of reperfusion ($n = 5$ for group). Sham surgery only = 6	Either 30 min. or 2 hrs of MCAO			Authors found major changes of GDNF family signaling in the forebrain, regulated mainly through altered receptor levels, in the post-ischemic phase that could enhance neuroprotective and neuroregenerative responses both to endogenous and exogenous GDNF ligands.
	[334]	Adult spontaneously hypertensive rats.		1 hr transient MCAO	i.c.v. IGF-1 and GDNF infusions by osmotic minipump. BrdU (i.p.; twice a day; 50 mg/kg starting at 1 day after MCAO or sham surgery	1 day before of MCAO	MCAO-induced progenitor cell proliferation in the ipsilateral DG was significantly increased by i.c.v. infusion of IGF-1 and GDNF compared to vehicle.
	[335]		1° GDNF ($n = 9$) vehicle ($n = 9$) 2°: GDNF ($n = 10$) vehicle ($n = 10$)	2-hr MCAO	1° experiment: Recombinant human GDNF (1.5 mcg/μL) or vehicle was infused intrastriatally *via* osmotic minipumps. BrdU, 50 mg/kg was injected i.p. 3 times at 2-hr intervals on day 7 2° experiment: BrdU was given 3 times daily for 1 week starting 6 days after the 2-hr MCAO.	1° experiment: after MCAO for 7 days. 2° experiment: from day 13 to day 26 after MCAO.	GDNF infusion increased cell proliferation in the ipsilateral SVZ and the recruitment of new neuroblasts into the striatum after MCAO and improved survival of new mature neurons. The GDNF receptor GFRalfa1 was up-regulated in the SVZ 1 week after MCAO and was coexpressed with markers of dividing progenitor cells.

Continued

Table 2 Continued

Neurotrophins	Study reference	Animal model	Sample size (treatment group/control group)	Type of study (focal or global ischemia)	Treatment	Time of administration	Results
	[266]	Rat	Group 1, control = 7; group 2, MSC-EGFP = 6+3; group 3, MSC-GDNF = 7+3; group 4, MSC-BDNF = 7+3; group 5, MSC-CNTF = 7+3; group 6, MSC-NT3 = 7+3	90 min. transient MCAO	5 × 105 adenoviral vector for each EGFP, GDNF, BDNF, CNTF, NT3 genes transfeted-human MSC were intrastriatal injected.	After MCAO	Rats that received MSC-BDNF or MSC-GDNF showed significantly more functional recovery and reduced ischemic damage on MRI than controls Rat that received MSC-CNTF or MSC-NT3 showed neither functional recovery nor ischemic damage reduction compared to controls.
	[336]	Rat	Control = 6+6+4; hMSC = 6+6+4; GDNF hMSC = 6+6+4+4+4+4 Normal = 4	Permanent MCAO	GDNF-hMSCs were i.v. infused (1.0×10^7)	3 hrs later MCAO	Rats receiving GDNF-hMSCs or hMSCs exhibited increased recovery from ischemia compared with the control group, but the effect was greater in the GDNF-hMSC group.
EGF	[342]	Rat	50	Transient global cerebral ischemia was induced by cardiac arrest, and resuscitation was started at 7 min.	1° xperiement: I.c.v. EGF injection by osmotic minipump (0.5 μl/hr, at 400 or 40 ng/d). Albumin was in 3- and 30-fold molar excess compared with EGF; 2° experiment: EGF (400 ng/d)	1° experiment: 2 days after ischemia for 7 days; 2° experiment: 21 days after ischemia for 7 days	EGF mRNA was not detected in either the control or the postischemic rat brain. Heparin-binding EGF-like GF (HB-EGF) mRNA expression was rapidly increased in the CA3 sector and the DG of the hippocampus, cortex, thalamus, and cerebellar granule and Purkinje cell layers. EGF receptor mRNA also showed an increase in the CA3 sector and DG.
	[343]	Mice		20 min. MCAO			I.c.v. EGF and albumin augments 100-fold neuronal replacement in the injured striatum after cerebral ischemia. Newly born immature neurons migrate into the ischemic lesion and differentiate into mature parvalbumin-expressing neurons, replacing more than 20% of the interneurons lost by 13 weeks after ischemia and representing 2% of the total BrdU-labeled cells.

Continued

Table 2 Continued

Neurotrophins	Study reference	Animal model	Sample size (treatment group/control group)	Type of study (focal or global ischemia)	Treatment	Time of administration	Results
	[344]	Rat		Transient forebrain ischemia bilateral: coagulation of vertebral arteries and bilaterally 6 min. BCCAO.	A cocktail of FGF-2 and EGF was infused i.c.v. bilaterally by osmotic minipumps (1.0 μl/hr, resulting in a delivery of 1440 ng of each GF per day, per brain for 3 days)	Days 2–5 after ischemia	Endogenous progenitors proliferate in response to ischemia and subsequently migrate into the hippocampus to regenerate new neurons. I.c.v. infusion of GFs markedly augments these responses, thereby increasing the number of newborn neurons.
	[296]	endothelin-1 (ET-1) rat model	22	MCAO *via* intracerebral microinjection of ET-1	Combined i.c.v. EGF/bFGF (0.48 μg/factor/day) *via* osmotic pump.	10 min. after ischemia.	EGF/bFGF substantially increased the infarct volume in ischemic animals. They induced increase in cell proliferation in the lateral ventricle 14 days after surgery and in the striatum.
	[345]	rat		80 min. MCAO	Either adenovirus-expressing HB-EGF (Ad-HB-EGF) or Ad-LacZ injected into the lateral ventricle on the ischemic side (1.1×10^{10} pfu/ml)	3 days after MCAO	There was no significant difference in infarct volume between the 2 groups. Treatment with Ad-HB-EGF significantly increased the number of $BrdU^+$ cells in the SVZ. $BrdU^+$ cells differentiated into mature neurons in the striatum on the ischemic side but seldom the cells given Ad-LacZ. Enhancement of angiogenesis at the peri-infarct striatum was also observed in Ad-HB-EGF–treated rats.
FGF	[292]	Rat		Permanent MCAO	bFGF (45 mcg/kg/hr) or vehicle were infused i.v. for 3 hrs.	30 min. after MCAO	After 24 hr, neurological deficits and infarct volume were significantly bFGF-treated animals compared to controls. Labeled bFGF crossed the damaged BBB to enter the ischemic (but not the non-ischemic) hemisphere
	[293]	Rat		Permanent MCAO	i.v. injection of bFGF, 150 μg/kg, or vehicle.	2 hrs after MCAO	Treatment with bFGF showed a significant improvement in functional tests, and a reduction of volume of cortical infarction
	[294]	Rat	Sham-bFGF/sham vehicle = 4–6/6; Stroke-BFGF/stroke vehicle = 5–6/6	MCAO	bFGF (0.5 mcg) or vehicle were administered intracisternally	24 and 48 hrs after MCAO	Infarct size did not differ among rats with or without bFGF treatment. bFGF increased cell proliferation in the ipsilateral SVZ and DG

Continued

Table 2 Continued

Neurotrophins	Study reference	Animal model	Sample size (treatment group/control group)	Type of study (focal or global ischemia)	Treatment	Time of administration	Results
	[295]	Rat		Transient MCAO	EGF+FGF-2 (each 1.44 ng/d) was continuously administered i.c.v. for 3 days	On day 1 of reperfusion	GFs infusion enhances post-ischemic progenitor cell proliferation by 5 days of reperfusion and neuronal maturation by 21 days of reperfusion in both the DG and SVZ in the adult rat brain.
	[297]	Rat	6 for group	2 hrs transient MCAO	Non-modified MSCs, FGF-2–modified MSCs with HSV-1 1764/-4/pR19/ssIL2-FGF-2, or PBS were administered intracerebrally.	24 hrs after transient MCAO	Animals with FGF-2–modified MSCs after stroke demonstrated significant functional recovery, reduction in infarction volume and higher FGF-2 production in treated brain compared with the other groups.
	[298]	Rat		Transient MCAO	A replication defective, recombinant adenovirus vector expressing FGF-2, was injected i.c.v.	2 hrs after MCAO	The treatment group showed significant recovery and decreased infarct volume compared with the vehicle-treated groups. FGF-2 gene transfer concentration was increased significantly not only in cerebrospinal fluid but also in cerebral substance in the lesioned and treated animals
BDNF	[278]	Rat	44	MCAO	NGF and BDNF were continuously delivered to the striatum at biologically active levels *via* recombinant (rAAV) gene	Transfer 4–5 weeks prior to 30 MCAO	Mild functional improvements 3–5 weeks after the insult, in agreement with a small but significant increase of the survival of dorsolateral striatal neurons.
	[270]	Rat	BDNF $n = 12$; vehicle $n = 13$	2-hr MCAO	Continuous i.v. infusion of BDNF (300 mg/kg per hr) for 3 hrs	30 min. after occlusion	Neurological deficit and infarct volume were improved in BDNF-treated animals *versus* controls. In these animals Bax-positive neurons were significantly reduced whereas Bcl-2–positive neurons were significantly increased in the penumbra.
	[276]	($nt4^{-/-}$) ($bdnf^{+/-}$) or wt mice	Mice lacking both alleles for neurotrophin-4 ($nt4^{-/-}$) or deficient in a single allele for brain-derived neurotrophic factor ($bdnf^{+/-}$)	1-hr MCAO			($nt4^{-/-}$) and ($bdnf^{+/-}$) mice exhibited larger cerebral infarcts compared to wild-type Lesions were larger in $nt4^{-/-}$ mice after MCAO

Continued

Table 2 Continued

Neurotrophins	Study reference	Animal model	Sample size (treatment group/control group)	Type of study (focal or global ischemia)	Treatment	Time of administration	Results
	[277]	Rat	GFPrAAV = 10; BDNF-rAAV = 14	Global forebrain ischemia by BCCAO carotid arteries combined with hypotension	Unilateral injections into two sites in the DG hilus of rAAV containing either the GFP gene (GFPrAAV) or the genes for GFP and BDNF (BDNF-rAAV), under control of the rat NSE promoter	5 weeks before ischemia	In treated animals BDNF induced a functional response that inhibits the formation of new dentate granule cells triggered by global forebrain ischemia.
	[284]	Rat		Permanent MCAO	i.v. injection of 50 μg/rat of either BDNF alone or BDNF as a conjugate with the murine OX26 MAb to the rat TfR	After MCAO	BDNF resulted in both a reduction in stroke volume and an improvement in functional outcome following delayed i.v. administration in regional brain ischemia, provided the neurotrophin is conjugated to a BBB molecular Trojan horse as BDNF-MAb conjugate.
	[282]	$BDNF^{+/-}$ mice and wt		40 min. MCAO			$BDNF^{+/-}$ mice had a significantly improved motor function compared with wild-type mice. There was no effect of BDNF reduction on infarct volume. Neurogenesis is induced following experimental stroke, and in the striatum of $BDNF^{+/-}$ mice significantly increased numbers of neuroblasts compared with wild-type.
	[279]	Rat		Distal MCAO			Authors found permanent reduction of BDNF and its full-length receptor, TrkB, in the infarcted core and a transient increase in BDNF immunoreactivity in the internal region of the border of the infarct, suggesting that BDNF regulates its full-length TrkB receptor in cortical neurons of the penumbra area and prevents their death.

MCAO: middle cerebral artery occlusion; i.v.: intravenously; s.c.: subcutaneously; i.p.: intraperitoneally; BBB: blood brain barrier; SVZ: subventricular zone; DG: dentate grisus; i.c.v.: intracerebroventricularly; NSCs: neural stem cells; BCCAO: bilateral common carotid artery occlusion; SN: substantia nigra.

In contrast, intracisternal administered bFGF in a rat stroke model does not seem to influence infarct size, but did enhance neuronal sprouting, synapse formation and progenitor cell proliferation and differentiation [291–294]. Interestingly, bFGF mRNA expression significantly increases as early as 60 min. following ischemia and remains elevated for up to 2 weeks after onset in a focal cerebral ischemia induced by transient occlusion of the middle cerebral and both common carotid arteries [285]. Moreover, a combined treatment with intraventricularly infused EGF and FGF-2 in adult spontaneously hypertensive rats [295] or in ischemic rats by MCAO occlusion [296] showed that this combined treatment further increases ischemia-induced endogenous neurogenesis and cell maturation. Also, administration of BMSCs engineered to produce FGF2 [297] and recombinant adenovirus vector expressing FGF2 decreased infarct size [298], supporting a neuroprotective role and a neurogenesis-stimulating property for this GF, taking advantage of a less invasive administration method.

Current evidence and future perspectives

The results of experimental studies support the implementation of a phase III clinical trial of bFGF in human beings. However, a North American phase II/III trial was halted after review of data from 300 patients with acute ischemic stroke owing to higher mortality rates in the treated group compared with the control group [299]. The continuing European-Australian phase II/III randomized trial in 286 acute ischemic stroke patients confirmed that intravenous administration of Trafermin (bFGF, 5 or 10 mg) or placebo intravenously infused over 24 hrs did not produce any significant neuroprotection, causing instead dose-dependent hypotension and an increased mortality rate in treated patients [300].

Recently, one study investigated bFGF serum levels in 30 patients with acute cerebral infarction and found that serum bFGF increased significantly after cerebral infarction in comparison with a control group, peaked on day 3, and remained significantly elevated until day 14 after stroke. In this study, it was also found that bFGF levels correlated with infarct size and clinical prognosis [301]. Thus, because of the number of side effects and increased mortality reported in the first clinical studies, further experimental studies are necessary to assess whether it is possible to achieve a pharmacologically significant therapeutic level in brain, minimizing peripheral side effects.

Insulin-like growth factor 1

IGF-1, a peptide produced primarily in the liver and brain, is best characterized as a mediator of growth hormone actions because of its important role in tissue growth regulation. A less-recognized action of serum IGF-1 is its multipotent neurotrophic activity [302]. Circulating IGF-1 can cross the BBB [303–306] and modulates brain function in many different ways [306–308]. It is a well known angiogenic factor [309] that also plays an essential role in normal growth and brain development [310], other than in modulation of vessel formation during brain development [311], and possibly in age-related changes in the brain vasculature [312]. It was also reported to enhance endothelial function through its anti-inflammatory and anti-apoptotic properties [313].

In past years, it was supposed that both brain-derived and circulating IGF-1 acted as a neuroprotective signal against acute ischemic brain injury [314, 315]. Actually, the role of IGF-1 in ischemic stroke is controversial, even in consideration of the wide variability of serum IGF-1 levels among individuals [316]. For instance, serum IGF-1 levels in human beings may be depressed after acute stroke [314, 315], whereas increased IGF-1 levels have been detected proximal to ischemic lesions in rodent models [317], suggesting a protective role of IGF-1 [302]. Many later studies showed that IGF-1 administration protects adult brain from experimental ischemia [318–320]. Intranasal (IN) delivery of rhIGF-I in a MCAO rat model significantly reduced the infarct volume *versus* controls, and improved all neurologic deficits [321].

In permanent distal MCAO in mice, long-term cerebral IGF-1 overexpression by the AAV transduction system through stereotaxic injection significantly improved motor performance, reduced the volume of cerebral infarction, and increased neovessel formation in the peri-infarct and injection needle tract area. Increased vascular density was associated with increased local vascular perfusion.

AAV-IGF-1 treatment was also observed to enhance neurogenesis in the SVZ [322]. Moreover, intravenous infusion of IGF-1 significantly decreased both lesion volume and apoptosis in diabetic animals after MCAO [323]. Therefore, serum IGF-1 would be expected to improve stroke outcome.

Since Schwab *et al.* observed decreased plasma levels of IGF-I and its receptor, IGFBP-3 in patients with acute stroke over a 10-period after onset of symptoms [314], the pathophysiological mechanism and the time course of IGF-1 levels after ischemic stroke must be better clarified.

Current evidence and future perspectives

To date, no clinical trials have been implemented to assess the benefit of IGF-1 on recovery in stroke patients. However, while the experimental studies to date are promising, a cautious approach to clinical application is needed since it is not clear how IGF-1 exerts its neuroprotective role.

Glial cell line-derived neurotrophic factor

Glial cell line-derived neurotrophic factor (GDNF) is a potent neurotrophic factor, acting mostly through the extracellular glycosylphospatidylinositol (GPI)-linked receptor, GFR-1 and the transmembrane tyrosine kinase, c-Ret. It was purified and characterized in 1993 as a GF promoting survival and differentiation of embryonic midbrain dopaminergic neurons [324, 325]. Subsequently, GDNF was shown to be a very potent trophic factor for spinal motoneurons [326] and central noradrenergic neurons [327], and to promote survival and differentiation of many peripheral neurons such as sympathetic, parasympathetic, sensory and enteric neurons [328]. Therefore, this trophic factor raised the

expectation that it might have potential as a therapeutic agent for the treatment of neurodegenerative diseases.

In MCAO rats, topical application of GDNF reduced both infarct size and brain oedema [329–331]. The authors suggested that the ameliorative effect of GDNF is related to its anti-apoptotic properties [329]. GDNF receptor expression was up-regulated in the penumbral area and GDNF delivery seemed to improve short-term neuronal survival in MCAO rats [332, 333]. Several findings suggest that GDNF may affect neurogenesis after stroke. Exogenous infusions of GDNF increased focal ischemia-induced cell proliferation of the ipsilateral DG of the subgranular zone (SGZ) in adult rats [334]. More recently, Kobayashi *et al.* [335] infused GDNF intra-striatally into rats after a 2-hr MCAO to clarify whether GDNF delivery during the first week after MCAO affects cell proliferation in the SVZ and recruits neuroblasts into the damaged striatum. The results of this study showed that GDNF infusion promoted survival of new neurons during the post-ischemic phase without influencing the speed of neuronal maturation. Since GFRα_1 levels were also elevated in the SVZ one week after stroke, the authors suggested that the effects of GDNF on cell proliferation are mediated by receptors present on stem cells.

Intravenously administered GDNF-gene modified hMSCs in MCAO rat models reduced the infarct volume, improved behavioural performance, and increased GDNF levels in the infarcted hemisphere [266, 336]. Intrastriatal injections of recombinant lentivirus (rLV) or adenovirus (rAAV) carrying the GDNF gene (rLV-GDNF or rAAV-GDNF) were performed in rats after transient MCAO. Both routes of GDNF gene delivery effectively promoted high striatal GDNF levels, which persisted several weeks after stroke. In contrast with previous experiments, the treatment did not increase neuron survival or improve functional recovery. The authors proposed that the effect of GDNF is complex and may depend on several factors, primarily protein levels, degree and pattern of receptor expression on cells in the damaged areas, route of delivery, type of cells transduced, and GDNF's actions on neuronal transmission at the site of delivery [333].

Current evidence and future perspectives

Despite pre-clinical studies demonstrating that GDNF improves neurogenesis and cell survival and differentiation, these effects are controversial. Thus, it is too early to test this neurotrophin in clinical studies, and more experimental studies should be performed to explain the heterogeneous results from previous studies.

Heparin-binding EGF-like growth factor

Heparin-binding EGF-like growth factor (HB-EGF) is a member of the EGF family, originally described as a macrophage-derived mitogenic and chemotactic factor of vascular smooth muscle cells [337]. HB-EGF is synthesized in a membrane-anchored form (proHB-EGF) and released as a secreted form, soluble HB-EGF [338]. HB-EGF is widely distributed in neurons and neuroglia throughout rat brain [339]. A number of studies demonstrated its neurotrophic activities *in vitro,* such as survival-promoting and neurite outgrowth effects [340], and neuroprotective effects against anoxia and nitric oxide toxicity [341]. *In vivo*, both HB-EGF and EGFR mRNAs were up-regulated following ischemia in rats, mostly in the hippocampal CA3 sector and the DG [342]. Teramoto *et al.* [343] first demonstrated that intraventricular administration of EGF amplifies proliferation of neural progenitors in the SVZ and the replacement of striatal interneurons after MCAO. Nakatomi *et al.* (2002) showed that a combined treatment with EGF and bFGF increased the number of $NeuN^+$ neurons 4 weeks after global ischemia in rats, confirming the role of this neurotrophin in neurogenesis [344]. This finding was recently confirmed by Baldauf *et al.* [296] who found that EGF/bGF infusion into the lateral ventricle in a model of MCAO rats, induced by endothelin injection, influences neurogenesis and cell proliferation in the striatum, supporting the notion that administration of these GFs accelerates cell migration from the ventricle and enhances proliferation [296]. Surprisingly, chronic administration of EGF/bFGF results in an enlarged infarct volume, raising some doubts about the treatment schedule and time of neurotrophin administration [345]. Watanabe injected adenovirus transferring the HB-EGF gene into the lateral ventricle on the ischemic side in a transient cerebral ischemia rat model and observed not only increased neurogenesis, but also enhanced angiogenesis in the peri-infarct striatum on day 8 in HB-EGF-treated rats, improving neurological functional recovery [298]. The proposed hypothesis was that HB-EGF promotes angiogenesis by inducing VEGF secretion from vascular smooth muscle cells, which enhances proliferation of endothelial cells.

Current evidence and future perspectives

Experimental models show that post-ischemic EGF/bGF intraventricular administration and gene therapy using the HB-EGF gene improves neurogenesis and angiogenesis. However, there are doubts about treatment schedules and the appropriate time of administration. Additional pre-clinical studies are necessary to better ascertain dosage, timing and the best method of administration.

Discussion

Post-stroke brain remodelling proceeds through several different mechanisms including neurogenesis, angiogenesis, axonal plasticity and white matter changes. Post-stroke neurogenesis and angiogenesis consists of different steps as well. Endogenous neurogenesis in response to brain ischemia requires neuronal stem cells and progenitor cells proliferation, differentiation of neuronal progenitor cells and migration of neuroblasts to the peri-ischemic area where they integrate into the parenchymal tissue as mature neurons. Angiogenic responses to pathophysiological conditions include endothelial cells proliferation and migration, tube formation

and branching and anastomosis [346]. Our increasing understanding of the complex mechanism underlying endogenous responses to brain ischemia may identify therapeutic options that will promote post-stroke brain recovery.

GFs are normally expressed in adult brain [108, 155] and are upregulated in response to brain ischemia [36, 40, 110, 113, 184, 242, 246, 247]. Moreover, knockout mouse studies confirm that deficiencies in select GFs impairs the response to brain ischemia, leading to a larger infarct size and poorer clinical outcome [41, 91].

Haematopoietic GFs, through their pleiotropic effects, play a significant role in enhancing ischemic brain remodelling at different levels, and counteract the ischemia-induced phenomena that contributes to brain damage after stroke, such as inflammation and oedema.

G-CSF plays a significant role in post-stroke neurogenesis by mobilizing endogenous $CD34^+$ stem cells and promoting their homing to the site of injury [49, 50] and in promoting cell proliferation from the SGZ of DG [65–68] as well as reducing post-stroke brain damage. A significant reduction in brain oedema and BBB damage after G-CSF administration is probably mediated by the suppression of injury-induced pro-inflammatory responses and T-cell infiltration [45–47]. EPO promotes post-ischemic brain remodelling, influencing stem cell differentiation and playing an angiogenic role [88–91, 129, 131]. Additionally, EPO regulates the expression of genes involved in apoptosis [115–118], to reduce the production of pro-inflammatory cytokines [120] and brain oedema [123].

GM-CSF results in with improved brain hemodynamic parameters [161], favours proliferation and structural changes in intraparenchymal vessels after stroke [158, 163], enhances leptomeningeal collateral growth [163] and counteracts metabolic damage subsequent to hypotension [162]. In addition to this, GM-CSF administration results in a positive modulation of apoptosis-related genes leading to a significant decrease in neuronal apoptosis [155, 160]. VEGF and its receptor (VEGFR-2) play a significant role in the initial phase of angiogenesis. Angiopoietins are involved in the maturation, stabilization and remodelling of vessels, and VEGF overexpression resulted in a higher capillary density but not a corresponding increase in blood flow [199]. Moreover, VEGF counteracted apoptosis and reduced brain oedema.

Taken together, the results of pre-clinical studies show that bEGF, VEGF, G-CSF, EGF, FGF-2, IGF and GDNF [343, 334] are upregulated after ischemia [294, 317, 347–350] and stimulate both *in vitro* and *in vivo* neurogenesis and angiogenesis. In particular, in experimental models, they were shown to contribute to infarct size and brain oedema reduction, cell proliferation, and improved survival of new mature neurons, apoptosis reduction, increased neovessel formation and improved clinical outcome (Tables 1 and 2). bFGF, VEGF, EPO and G-CSF have all shown potential effectiveness in stroke recovery in experimental models.

On the basis of these promising results, some of these factors were tested in clinical trials. Completed EPO trials [138, 139] involving patients with hyperacute stroke (treatment within 8 hrs of symptom onset) reported a positive, but insignificant [138] tendency, both in terms of infarct size reduction and clinical outcome, suggesting a possible neuroprotective effect in the treatment arm. The results from three G-CSF trials in stroke patients showed that it is safe, well-tolerated, and that its administration results in mobilization of mobilizing bone marrow $CD34^+$ stem cells [69]. Despite this, the results on clinical outcome from two clinical trials involving patients with recent stroke were uncertain suggesting a positive, but non-significant effect on 90-day disability (BI) [69], neurologic function (NIHSS) after 20 days [71] and long-term outcome (12-month assessment) [70].

bFGF administration resulted in dose-dependent hypotension and increased the mortality rate in treated patients compared with the control group [300].

As a consequence of the methodological heterogeneity between different trials and the small patient populations, there is a substantial lack of evidence for the administration of GFs to patients with stroke [72]. Thus, although data from the existing trials suggest that EPO and G-CSF are safe and well-tolerated and may have a positive effect in terms of neuroprotection (reduced infarct size after EPO administration) and neuronal repair (G-CSF), further studies are necessary to assess the safety and efficacy of GF use in stroke patients.

The failure of clinical trials with bFGF raises questions about the importance of the BBB in achieving a pharmacologically significant therapeutic level of GFs in brain, and minimizing side effects in the peripheral tissues. For instance, intranasal delivery may be considered as a practical, non-invasive method of bypassing the BBB to deliver therapeutic agents to the brain [351]. Avoiding side effects in peripheral tissues is obviously an important issue. Additionally, the development of structural and functional variants of EPO with reduced or absent haematopoietic functions represents a challenge in stroke treatment [139].

The optimal timing of administration of GFs is still unclear, since most published studies on stroke patients focus on recent stroke (enrolment from 7 to 30 days from stroke onset), and data on their use in acute and chronic stroke are lacking. For instance, Shyu *et al.* [70] reported a greater clinical improvement in the subgroup ($n = 4$) of patients who received G-CSF within 24 hrs, but this finding should be confirmed. Also, the safety of GFs administered in this subgroup of patients should be assessed. With regard to VEGF, it was more effective over 48 hrs, so as to avoid the increased risk of brain oedema in the acute phase [200, 203]. However, IGF-1 was not beneficial in the acute phase [302]. With regard to G-CSF, a few pre-clinical studies [261, 352] suggest a possible positive effect in chronic stroke.

The current uncertainty regarding the use of GFs as stroke treatments derives from the fact that none of the trials definitively addressed the mechanisms by which GFs might work after stroke. Focusing on the potential mechanisms of action determined in pre-clinical studies will help investigators decide when to administer treatment relative to stroke onset.

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