Published in final edited form as:

Neuroscience. 2009 September 29; 163(1): 233–243. doi:10.1016/j.neuroscience.2009.05.050.

TGF α Induces Angiogenesis and Neurogenesis Following Stroke

Ronen R. Leker^{*,1,2}, Zsuzsanna E. Toth^{*,2,3}, Tal Shahar^{*,2}, Riccardo Cassiani-Ingoni², Ildyko Szalayova², Sharon Key², Andras Bratincsák², and Eva Mezey²

¹Department of Neurology, Peritz and Chantel Scheinberg Cerebrovascular Research Laboratory, Hebrew University-Hadassah Medical Center, Jerusalem Israel

²National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda MD

³Neuromorphological and Neuroendocrine Research Laboratory, Department of Anatomy, Histology and Embryology, Semmelweis University and the Hungarian Academy of Sciences, Budapest, Hungary

Abstract

The cytokine transforming growth factor α (TGF α) has proangiogenic and proneurogenic effects and can potentially reduce infarct volumes. Therefore, we administered TGF α or vehicle directly into the area surrounding the infarct in female mice that received gender-mismatched bone marrow transplants from GFP-expressing males prior to undergoing permanent middle cerebral artery occlusion. Newborn cells were tracked with BrdU labeling and immunohistochemistry at 90 days after stroke onset. We also studied the ingress of bone marrow derived cells into the ischemic brain to determine whether such cells contribute to angiogenesis or neurogenesis. Infarct volumes were measured at 90 days post stroke. The results show that TGF α led to significant increments in the number of newborn neurons and glia in the ischemic hemisphere. TGF α also led to significant increments in the number of bone marrow derived cells entering into the ischemic hemisphere. Most of these cells did not label with BrdU and represented endothelial cells that incorporated into blood vessels in the infarct border zone. Our results also show that infarct size was significantly reduced in animals treated with TGF α compared with controls. These results suggest that TGF α can induce angiogenesis, neurogenesis and neuroprotection after stroke. At least part of the pro-angiogenic effect appears to be secondary to the incorporation of bone marrow derived endothelial cells into blood vessels in the infarct border zone.

Keywords

Stroke; Transforming Growth Factor Alpha; Neurogenesis; Angiogenesis

Transforming growth factor α (TGF α) is a pleiotropic cytokine that binds to the epidermal growth factor receptor (EGFR) to produce its downstream effects (Cameron et al., 1998, Irvin et al., 2003). Both TGF α and EGFR are present in the subventricular proliferative zone (SVZ) where they modulate the activity of neural stem cells (NSC) and neural progenitor cells (NPC)

1Current Address and Address for Correspondence: R.R. Leker MD, Stroke Service and the Peritz and Chantel Cerebrovascular Research Laboratory, Hadassah Ein Kerem P. O. Box 12000, Jerusalem 91120, Israel. Tel 972-2-6776945 Fax 972-2-6437782 Email leker@cc.huji.ac.il.

*These authors contributed equally to the work

Section Editor – Cellular Neuroscience Prof. Menahem Segal

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

(Kornblum et al., 1997). Exogenously applied TGF α increases NSC number and survival and can induce differentiation to neural and glial fates (Cameron et al., 1998, Cooper and Isacson, 2004). TGF α also reduces the infarct size after ischemic injury; an effect that is also mediated by EGFR (Justicia and Planas, 1999).

Neural stem cells interact with endothelial cells in a specialized vascular-neural stem cell niche (Palmer et al., 2000). This interaction is important for their survival and differentiation and may be impaired in adult animals leading to a suboptimal response following an ischemic insult (Edelberg and Reed, 2003, Enwere et al., 2004). We recently showed that bone marrow derived endothelial cells (BMDEC) significantly contribute to angiogenesis in the ischemic brain (Toth et al., 2008) but it is unknown if TGF α can influence this process. To examine whether TGF α can induce angiogenesis, neurogenesis and entry of BMDEC into the brain we used EGFP chimeric animals that underwent permanent MCAO (PMCAO) and were later treated with vehicle or TGF α .

Materials and Methods

Preparation of the mice and surgery

All experiments were approved by the institutional animal care and use committee and were conducted according to NIH guidelines. Female 4-6 weeks old C57B mice (n=30) were subjected to irradiation (2 $\times$ 450 rad) to deplete their own bone marrow (BM). The same day following the second irradiation they were transplanted with bone marrow (Mezey et al., 2000) generated from male mice that express green fluorescent protein (GFP) in all of their cells (with the exception of erythrocytes) and kept in sterile environment for 10 days (Figure 1). After recovery, they were subjected to distal permanent middle cerebral artery occlusion (PMCAO) as described before (Leker et al., 2002). Briefly, animals are anesthetized and placed in a stereotaxic head holder. The left distal MCA is exposed through a craniotomy and the dura is reflected off the artery. The MCA is then occluded by electrocoagulation, a piece of gelfoam is placed on the exposed brain and the muscle and skin are sutured. This model results in cortical injury limited to the frontal and parietal cortex and spares subcortical structures. A separate group of sham-stroke animals (n=4) was used as controls. In these animals, the MCA was exposed but not occluded. Immediately after induction of ischemia, a cannula was inserted into the brain adjacent to the infarct border using the following stereotaxic coordinates: 1.00 mm posterior to the bregma; 2.50 mm lateral; 3.00 mm dorsal ventral from the surface of the skull. Cannulas were attached via sterile polyethylene tube to a sterile ALZET 1002 mini-osmotic pump and were fixed to the skull with sterile acrylic dental cement. The pumps contained either TGF α (20ng/ml) or commercially available sterile artificial CSF (Harvard Apparatus Holliston, MA) containing the following ion concentrations in mM: Na 150; K 3.0; Ca 1.4; Mg 0.8; P 1.0; Cl 155 (n=15/group). Mice were active for 2 weeks after which the cannulas were removed and the animals were allowed to survive. The specific dose and coordinates were used because according to previous studies they yielded significant increments in angiogenesis and neurogenesis respectively (Fallon et al., 2000, Justicia et al., 2001, Cooper and Isacson, 2004). All mice were given BrdU (50mg/kg/bid IP) on days 1-5 post PMCAO to follow cell proliferation.

Immunohistochemistry

Mice were perfused 90 days after surgery using 4% paraformaldehyde through the ascending aorta. Following perfusion, the brains were taken out and processed using cryoprotection achieved by increasing concentration of sucrose. The brains were then frozen on dry ice and serial sections were cut at 10 μ m thickness and mounted on positively charged microscope slides. The slides were kept at -80°C until used. Immunohistochemistry with double and triple

staining was performed to visualize GFP as well as endothelium and neural lineage specific markers as detailed in Table 1.

The perfused sections were washed in PBS three times for three minutes, microwaved for antigen retrieval in 10mM Citric acid buffer (pH:6.0) for 5 min after the liquid started to boil and then cooled at RT for 30 min. Following pretreatment, the sections were blocked with 1X Universal Blocking Reagent (Biogenex, San Ramon, CA) for 10 min. The primary antibody was applied according to Table 1, diluted in 1% BSA containing 0.25% Triton-X 100 and followed by blocking endogenous peroxidase activity using 3% of H₂O₂ for 15 min. In case of double staining with a second tyramine amplification step, we added 0.5% of Sodium-azide to the H₂O₂ solution in order to block the HRP still present from the first staining. Alexa-fluor dyes (Invitrogen) were used for regular double staining and DAPI (Sigma) was used to visualize nuclei.

Immuno-positive cells were counted in the area surrounding the infarct on slides between bregma +1.42 and bregma -0.8. Overall, 7 evenly spaced slices were counted for each brain between bregma +1.42 and bregma -0.8 and in each slice high power magnification fields in the entire area surrounding the infarct were counted (30 fields at $\times 400$). This area included mainly cortex and subcortical tissue but not striatum.

Slides were double and triple stained to evaluate cell fate and Z-section confocal microscopy (Olympus) was used to ensure colocalization in double staining. Double positive cells were counted in the area surrounding the infarct as detailed above. Data are presented as total number of cells in the counted slides.

Y chromosome hybridization

To further confirm the origin of the GFP in a few animals we colocalized Y chromosome in the same cells as GFP as follows: sections were washed in PBS (pH: 7.4) three times for 3 min, rinsed in distilled water and incubated in 1X Universal Blocking Reagent for 10 min. The sections were then incubated in rabbit anti-GFP antibody (1:40,000, Molecular Probes, Eugene, OR) for 1h at room temperature. The endogenous peroxidase activity was blocked with a 3% hydrogen peroxide and following PBS washes the secondary antibody - an anti-rabbit HRP polymer conjugate (SuperPicture, Zymed Laboratories Inc., South San Francisco, CA) - was applied undiluted for 30 minutes. The staining was then visualized using FITC conjugated tyramide at 1:10000 for 10 minutes at RT. To perform Y chromosomal FISH, the same sections were immersed in 10mM citric acid (pH 6.0) and microwaved in a kitchen microwave (GE, 700W) for 2 $\times$ 5 min at 50% power after the liquid started to boil. The water that evaporated was replaced with distilled water between and after the microwaving sessions and the sections were left in the solution to cool for 2 hours at RT. Microwave treatment inactivates any HRP activity that is present in the tissue - i.e. endogenous HRP and/or HRP incorporated in reagents used in previous steps (Toth and Mezey, 2007). The Y chromosomal hybridization was performed using a 1.5-kb RNA probe, (pY3531B) generated against a repeat sequence of the mouse Y chromosome that was labeled with digoxigenin using a labeling kit (Digoxigenin RNA Labeling Kit, Cat. # 1 175 025 - Roche Applied Sciences, Indianapolis, IN) (Mezey et al., 2000). After the hybridization step and several washes in SSC (for details see our website: <http://intramural.nimh.nih.gov/lcmr/snge/>), the digoxigenin was detected with an anti-digoxigenin antibody conjugated to horseradish peroxidase (1:600, Roche Applied Sciences, Indianapolis, IN, Cat No: 1120773391) and visualized using the TSA-Plus CY3 System (1:600, PerkinElmer Life Sciences, Boston, MA (now Invitrogen)). Determination of colocalization of the Y chromosome with GFP was performed by counting the cells manually in three different sections per animal, 5 animals per group and by two independent people using a DMI6000 Leica inverted fluorescence microscope.

Infarct size determination

At 90 days post stroke series of sections (at 200 μ m intervals) from each forebrain were stained with toluidine blue, photographed and using the NIH ImageJ program the area of the stroke and both hemispheres were measured. Because at that time the infarct tissue has already liquefied the lesion size was determined by subtracting the area of the lesioned hemisphere from that of the normal hemisphere (also excluding the ventricles). $\text{Size} = (\text{normal hemisphere size} - \text{injured hemisphere size}) / \text{normal hemisphere size} \times 100$.

Blood vessel counts

The area surface of vessels ($n=6/\text{group}$) was determined by staining for endogenous alkaline phosphatase using NBT/BCIP (Invitrogen). The slides were then scanned using the T3 Scanscope by Aperio Technologies. The images were then analyzed using the NIH ImageJ software as described and validated previously (Toth et al., 2008). Blood vessels were visualized with antibodies directed against specific endothelial markers ($n=6/\text{group}$) such as von-Willebrand factor and CD31 using immunohistochemistry with double and triple staining techniques as described above, on slides surrounding the infarct core between bregma +1.42 and bregma -0.8. Overall, 7 evenly spaced slices were counted for each brain between bregma +1.42 and bregma -0.8 and in each slice evenly spaced high power magnification fields in the entire area surrounding the infarct were counted (30 fields at $\times 400$). Because by 90 days post stroke the infarct core had already liquefied, the area forming the outer boundary of the brain represents the ischemic border zone that survived. This area included mainly cortex and subcortical tissue but not striatum.

Statistical evaluation

The data were analyzed using the Sigma-Stat software and one way ANOVA was applied. Comparisons between active treatment groups and vehicle were done with the Tukey, Holmes-Sidak or Bonferoni methods. P values of <0.05 were considered as statistically significant.

Results

TGF α increases influx of GFP+ cells into the injured brain

In these experiments, we used a dose of TGF α that was previously reported to produce significant increments in neurogenesis in models of Parkinson's disease and to significantly reduce infarct volumes after stroke (Fallon et al., 2000, Justicia et al., 2001, Cooper and Isacson, 2004). In sham operated control animals we could only detect a very low number of GFP expressing cells in the brain. Most of these cells expressed the microglia marker Iba1 and were evenly distributed in both hemispheres (data not shown). In contrast, we observed a significantly higher number of GFP+ cells in brains of PMCAO mice and most of these cells were located in the hemisphere harboring the infarct. To ensure that cells expressing GFP indeed originate from donor BM we used gender-mismatched BM transplants and FISH to determine the presence of a Y chromosome in GFP+ cells. TGF α treatment caused a 4 fold increase in the influx of GFP+ cells into the ischemic hemisphere in the brain (Figure 2) compared with vehicle control.

At 3 months post PMCAO most of the brain GFP+ cells expressed endothelial markers in the vehicle and treatment groups. Rare GFP+ cells colocalized with the astrocyte markers GFAP or S100 β and no GFP+ cells expressed the neuronal markers NeuN or Hu. There were no significant differences as to the percentage of CD31+/GFP+/Y+ cells ($>85\%$ of GFP cells), IBA1+ microglia (10%) or GFP+/GFAP+ cells between controls and treated animals ($<5\%$ of GFP cells). Of note, we did detect occasional Y+/GFP-/CD31+ ($\sim 10\%$ of Y+ cells) cells in the brains of both groups suggesting that sole reliance on GFP as a method to label progeny

may lead to an underestimation of the observed effect. These results suggest that TGF α can increase the influx of GFP+ bone marrow derived cells into the ischemic hemisphere. The results also suggest that bone marrow derived cells entering the brain do not transdifferentiate into neural cells in large numbers and that most express endothelial markers in the long term after stroke.

TGF α increases angiogenesis in the injured brain

Treatment with TGF α led to a 2.4 fold increase in the area covered by blood vessels surrounding the infarct (Figure 3) compared with vehicle controls. Most blood vessels in both groups had detectable GFP+/vWF+ cells in their walls (82% in the TGF group and 61.5% in controls) but the total number of GFP+ cells in the vessels was larger in the treatment group (Figure 2). Similar results were seen when CD31 was used as a vascular marker. Taken together these results suggest that TGF α has a proangiogenic effect in the ischemic hemisphere and that this effect is probably secondary to the influx of BMDEC into the brain because many of the GFP expressing endothelial cells were found in the blood vessels present at the infarct border zone (Figure 3B). However, because not all endothelial cells in some of the blood vessel expressed GFP it is possible that TGF α might have also increased blood vessel number by protection of existing vessels and that GFP+ BMDEC replaced dead cells in the walls of such vessels.

TGF α increase cell proliferation in the ischemic brain

Cell proliferation was studied with BrdU immunostaining following PMCAO. The number of BrdU positive cells was significantly increased by TGF α treatment in both hemispheres with a much larger increment in BrdU+ cells observed in the ischemic hemisphere. BrdU positive cells were significantly increased at the infarct border with TGF α (by ~2.5 fold) compared with vehicle treated animals (Figure 4). When we tried to co-localize GFP with BrdU we found less than 1% of cells that double labeled and most of those expressed endothelial markers. These results suggest that most bone marrow derived cells did not express BrdU in the brain suggesting that they either did not proliferate in the brain for long periods of time or that proliferating cells did not survive in the brain or that these cells proliferated so much as to dilute the signal to below detection capabilities. These data also suggest that the vast majority of cells that did proliferate outside of the brain then homed to the ischemic hemisphere expressed endothelial markers. These results also suggest that TGF α serves as a mitogen for endogenous neural stem cells and progenitors (GFP negative) that did incorporate BrdU or that it protects such cells from dying. These results also suggest that despite the potentially damaging effects of irradiation on neurogenesis (Monje et al., 2003) TGF α was capable of inducing a sustained increase in endogenous neurogenesis.

TGF α increases neurogenesis in the injured brain

To examine the fate of newborn cells in the brain we double labeled cells with BrdU and markers for neural stem cells and progenitors (nestin), mature neurons (NeuN) and astrocytes (GFAP). In control mice that were given artificial cerebrospinal fluid (ACSF) instead of TGF α following PMCAO, the percentages of BrdU+ cells that also co-expressed NSC, neuronal and glial markers were 4%, 0.18% and 58% respectively (Figure 5). Newborn cells expressing the NSC marker nestin were significantly more abundant in animals treated with TGF α (by 4 fold compared with controls; Figure 5). Most of these nestin expressing cells were localized at the infarct border or at the SVZ (Figure 5). Newborn GFAP-expressing astrocytes were the most abundant type of differentiated newborn cell and their numbers were significantly increased (1.2 fold) in TGF α treated animals compared with controls (Figure 4). GFAP+ cells that also expressed BrdU were localized both at the SVZ and at the infarct border (Figure 5). In contrast, newborn NeuN expressing neurons were extremely rare. Their number was significantly larger in animals treated with TGF α compared with controls (Figure 5) and

these cells were only present in the cortex a few cell layers away from the infarct border zone (Figure 5).

Interestingly, we observed a significant increase in the number of doublecortin positive (DCX+) migrating neuroblasts in the ischemic hemisphere in TGF α treated animals (by 2.4 fold; Figure 6). These cells form clusters at the SVZ and migrate in chains towards the infarct (Figure 6). Many of these cells migrate in close proximity to blood vessels (Figure 6). However, because we saw a limited increase in newborn neurons in the TGF α treated group (Figure 5), our data suggests that many of these DCX+ cells do not undergo terminal differentiation into more mature neurons or only do so after at later time points. These results suggest that under these experimental circumstances TGF α drives glial and neuronal differentiation to a limited albeit significant extent whereas the percentage of undifferentiated cells remained smaller in treated animals.

TGF α reduces infarct volumes after ischemic cortical lesions

Infarct size was measured at 90 days post stroke as hemispheric percentage and was significantly smaller (~50% reduction) in the animals that were treated with TGF α compared with controls (Figure 7).

Discussion

The main findings of the current study are that treatment with intra-parenchymal TGF α leads to increased influx of bone marrow derived cells into the brain after stroke and that these are mostly endothelial cells that incorporate into blood vessels and do not transdifferentiate to a neuronal fate. Furthermore, TGF α increases the number of BrdU expressing cells in the brain suggesting either a proliferative effect of TGF α on endogenous progenitors or a protective effect on endogenous progenitor cells with the net result in either case being an increase in the number of newborn cells (Cameron et al., 1998, Cooper and Isacson, 2004). Most of these progenitors differentiate into astrocytes but some of these cells express the migrating neuroblast marker doublecortin and their numbers are significantly larger in TGF α treated mice. TGF α treated animals also had smaller infarcts attesting to the neuroprotective properties of this cytokine.

To explore whether bone marrow cells could differentiate into neurons after stroke we used bone marrow from GFP expressing male donors that engrafted into recipient female mice prior to PMCAO. We then identified donor marrow cells as expressing GFP and/or the Y chromosome using sensitive detection methods as previously described (Toth et al., 2007). Using this system we could distinguish between newborn neural cells of bone marrow origin and those of endogenous CNS origin. Our results corroborate previous reports that found increased recruitment of bone marrow derived cells to the brain following injury. Treatment with intra-parenchymal TGF α led to significant further increments in the influx of bone marrow derived GFP+ cells into the brain after stroke. However, the current set of results argues against trans-differentiation of BM derived stem cells into neurons as an important mechanism to functional improvement in stroke (Ono et al., 2003, Massengale et al., 2005). Thus, despite a major increase in the influx of GFP+ BM derived cells into the injured brain with TGF α we could only identify isolated astrocytes in the brain that co-expressed GFP. In contrast, most BM derived GFP+ cells in the brain represented endothelial cells which were the prevalent GFP+ cell type at 3 month post-stroke (Toth et al., 2008). Nevertheless, we can not rule out a more significant contribution of BM derived neurogenesis in other circumstances, such as in different diseases and/or at different time points (Mezey et al., 2000, Mezey et al., 2003). Furthermore, less than 1% of all BrdU+ cells co-expressed GFP implying that most bone marrow derived cells did not further divide in the brain. Alternatively, we can not rule out the possibilities that BM-derived proliferating cells did not survive in the brain, that they divided

so many times as to completely dilute the BrdU signal to below detection levels or that these cells proliferated at a time point that was outside of BrdU administration.

Furthermore, it is possible that BM derived cells had a positive effect on endogenous neurogenesis in the brain as significant increments in the number of endogenous (GFP-) BrdU positive cells were observed in animals treated with intracerebral TGF α . This effect may be secondary to a direct proliferative effect of TGF α on endogenous cells or to a protective effect of TGF α on these cells preventing their death.

Importantly, TGF α was given for 2 weeks after stroke and the data presented here shows evidence of increase in the number of dividing cells at 90 days after stroke. This implies that early therapy may have long-term effects and suggests that similar strategies may be implied in humans in future trials.

SVZ cells are known to migrate out towards the infarct and accumulate at the infarct border after stroke (Leker et al., 2007). This observation was confirmed in the present set of experiments as TGF α led to increased accumulation of newborn cells at the infarct border towards which chains of DCX+ migrating neuroblasts were also seen.

We observed a statistically significant 2 fold increase in the number of neuroblasts in the ischemic hemisphere whereas only very few newborn neurons expressed NeuN. Newborn NeuN/BrdU double positive cells were observed a few cells layers away from the infarct border. This may suggest that the environment at the immediate infarct border prohibits neuronal differentiation whereas such differentiation may be possible in a more permissive environment. The factors responsible for such inhibition of neuronal differentiation are not known yet but pertinent candidates may include myelin associated proteins as well as semaphorins and plexins among others (Swiercz et al., 2002, Charrier et al., 2003, Emanuelli et al., 2003, Wang and Zhu, 2008). In any case, it is highly unlikely that this slight increase in neurons could be responsible for major clinical improvements after stroke. In contrast, most differentiated cells in animals treated with TGF α expressed glial markers and their relative numbers were 1.2 fold larger than in controls.

In the current study BrdU was administered for the first few days after stroke and the effects of treatment were then studied 90 days afterwards. Therefore, it is possible that the effects of treatment are actually underestimated in the current study as BrdU labeling may have been diluted over time. However, we and others (Thored et al., 2006, Leker et al., 2007, Zhang et al., 2007) have previously shown that cell migration and proliferation persists for long periods of time after stroke onset. Thus, in a double labeling experiment using IdU and CldU which can be identified by different antibodies we have shown that early proliferating cells at the SVZ continue to proliferate late after stroke onset at the infarct border and these cells are easily identified with the proper antibodies (Leker et al., 2007). Therefore, we believe that the data presented does indeed represent a true effect of TGF α on neurogenesis. Furthermore, it is well appreciated that BrdU may also label other proliferating cells in the brain such as reactive astrocytes and microglia and not only dividing progenitors. Thus, it is possible that some of nestin and GFAP positive cells that co-expressed BrdU at the infarct border represent proliferating reactive astrocytes that did not originate from SVZ neural progenitors. However, we believe that the demonstration of an increased number of doublecortin positive cells in treated animals as well as a clear gradient of BrdU+ cells from SVZ to the infarct area as shown in Figure 3 supports the contention that these cells primarily represent migrating newborn cells. This is especially true when considering that microglia and reactive astrocytes largely cease to proliferate at 90 days after stroke.

Importantly, we can not totally exclude the possibility that the increase in the number of doublecortin expressing cells in the TGF α group may represent a neuroprotective anti-

apoptotic effect that prevented the death of neural progenitors rather than a mitogenic effect on precursors. However, the net result is an increase in the number of these cells at the infarct border.

Similarly, the increased number of blood vessels observed with TGF α may be related to a vasoprotective effect of treatment and the presence of GFP+ cells in vessel walls at the infarct border zone may be due to replacement of injured native endothelial cells by circulating proliferating bone marrow derived endothelial cells. However, given that many blood vessels at the infarct border contained a large number of GFP+/Y+ and GFP+/BrdU+ double expressing cells in their walls (see Figure 1a), and the large difference in the total area covered by blood vessels in this region argue for a pro-angiogenic effect for TGF α . TGF α was previously related to angiogenesis in tumor biology (Tsai et al., 1995, Kaur et al., 2005). This may be induced by TGF α signaling via EGF receptor, which in turn induces vascular endothelial growth factor expression and ensuing angiogenesis (Tsai et al., 1995, Kaur et al., 2005). In particular TGF α may induce an increased influx of bone marrow originating cells into the brain by inducing chemo attractants to endothelial cells including VEGF (Gille et al., 1997) or by a direct effect on endothelial migration (Bull et al., 1993).

Angiogenesis is induced in the brain shortly after ischemic onset and is driven by multiple genetic alterations (Hayashi et al., 2003). The mechanisms responsible for angiogenesis were recently reviewed by Beck and Plate (Beck and Plate, 2009) and include signaling via VEGF and angiopoietin receptors (Beck et al., 2000, Ferrara, 2000, Zhang et al., 2002, Beck and Plate, 2009), as well as an increase in concentrations of several other pro-angiogenic factors including platelet derived growth factor (Hayashi et al., 2007), erythropoietin (Wang et al., 2004, Li et al., 2007), transforming growth factor beta (Krupinski et al., 1996) and fibroblast growth factor (Carmeliet, 2000) among others. The end result involves induction of proliferation of brain derived endothelium (Beck and Plate, 2009). Here we describe a novel pathway of TGF α induced angiogenesis by increased recruitment of endothelial bone marrow derived cells into newly formed cerebral blood vessels. A similar mechanism was also observed when animals were treated with systemic infusion of granulocyte colony stimulating factor (Borlongan and Hess, 2003, Six et al., 2003, Willing et al., 2003, Sehara et al., 2007, Toth et al., 2008). Thus the mechanism of recruitment of bone marrow derived cells into the cerebral vasculature and formation of new blood vessels may be more general and common than previously appreciated.

Importantly, TGF α was previously found to reduce infarct volumes in injury models (Justicia et al., 2001). These neuroprotective effects are driven by anti-apoptotic and anti-inflammatory mechanisms (Justicia and Planas, 1999). Our results show that even when administered directly into the tissue surrounding the infarct core this protective effect is maintained.

In conclusion, TGF α had positive effects on lesion size and on both angiogenesis and neurogenesis following stroke. Therefore, it is suggested that it can be used to improve outcome after stroke.

Acknowledgments

The authors would like to thank Dr. Alfredo Molinolo for his assistance with the slide scanner and Wayne Rasband for his help with customization of the NIH Image software.

Dr. Leker was supported in part by the Chantel and Peritz Scheinberg Cerebrovascular Research Fund and by the Sol Irwin Juni Trust Fund

List of Abbreviations

TGF α

	transforming growth factor alpha
GFP	green fluorescent protein
BrdU	bromo-deoxy-uridine
SVZ	subventricular zone
NSC	neural stem cells
NPC	neural progenitor cells
EGF	epidermal growth factor
BMDEC	bone marrow derived endothelial cells
PMCAO	permanent middle cerebral artery occlusion
MCA	middle cerebral artery
BM	bone marrow
DCX	doublecortin
GFAP	glial fibrillary acidic protein
NeuN	neuronal nuclear antigen

References

- Beck H, Acker T, Wiessner C, Allegrini PR, Plate KH. Expression of angiopoietin-1, angiopoietin-2, and tie receptors after middle cerebral artery occlusion in the rat. *Am J Pathol* 2000;157:1473–1483. [PubMed: 11073808]
- Beck H, Plate KH. Angiogenesis after cerebral ischemia. *Acta Neuropathol.* 2009
- Borlongan CV, Hess DC. G-CSF-Mobilized human peripheral blood for transplantation therapy in stroke. *Cell Transplant* 2003;12:447–448. [PubMed: 12911132]
- Bull DA, Seftor EA, Hendrix MJ, Larson DF, Hunter GC, Putnam CW. Putative vascular endothelial cell chemotactic factors: comparison in a standardized migration assay. *J Surg Res* 1993;55:473–479. [PubMed: 8231165]
- Cameron HA, Hazel TG, McKay RD. Regulation of neurogenesis by growth factors and neurotransmitters. *J Neurobiol* 1998;36:287–306. [PubMed: 9712310]
- Carmeliet P. Fibroblast growth factor-1 stimulates branching and survival of myocardial arteries: a goal for therapeutic angiogenesis? *Circ Res* 2000;87:176–178. [PubMed: 10926865]

- Charrier E, Reibel S, Rogemond V, Aguera M, Thomasset N, Honnorat J. Collapsin response mediator proteins (CRMPs): involvement in nervous system development and adult neurodegenerative disorders. *Mol Neurobiol* 2003;28:51–64. [PubMed: 14514985]
- Cooper O, Isacson O. Intrastriatal transforming growth factor alpha delivery to a model of Parkinson's disease induces proliferation and migration of endogenous adult neural progenitor cells without differentiation into dopaminergic neurons. *J Neurosci* 2004;24:8924–8931. [PubMed: 15483111]
- Edelberg JM, Reed MJ. Aging and angiogenesis. *Front Biosci* 2003;8:s1199–1209. [PubMed: 12957863]
- Emanuelli C, Schratzberger P, Kirchmair R, Madeddu P. Paracrine control of vascularization and neurogenesis by neurotrophins. *Br J Pharmacol* 2003;140:614–619. [PubMed: 12970083]
- Enwere E, Shingo T, Gregg C, Fujikawa H, Ohta S, Weiss S. Aging results in reduced epidermal growth factor receptor signaling, diminished olfactory neurogenesis, and deficits in fine olfactory discrimination. *J Neurosci* 2004;24:8354–8365. [PubMed: 15385618]
- Fallon J, Reid S, Kinyamu R, Opole I, Opole R, Baratta J, Korc M, Endo TL, Duong A, Nguyen G, Karkehabadhi M, Twardzik D, Patel S, Loughlin S. In vivo induction of massive proliferation, directed migration, and differentiation of neural cells in the adult mammalian brain. *Proc Natl Acad Sci U S A* 2000;97:14686–14691. [PubMed: 11121069]
- Ferrara N. Vascular endothelial growth factor and the regulation of angiogenesis. *Recent Prog Horm Res* 2000;55:15–35. [PubMed: 11036931]discussion 35-16
- Gille J, Swerlick RA, Caughman SW. Transforming growth factor-alpha-induced transcriptional activation of the vascular permeability factor (VPF/VEGF) gene requires AP-2-dependent DNA binding and transactivation. *Embo J* 1997;16:750–759. [PubMed: 9049304]
- Hayashi T, Noshita N, Sugawara T, Chan PH. Temporal profile of angiogenesis and expression of related genes in the brain after ischemia. *J Cereb Blood Flow Metab* 2003;23:166–180. [PubMed: 12571448]
- Hayashi T, Wang XQ, Zhang HZ, Deguchi K, Nagotani S, Sehara Y, Tsuchiya A, Nagai M, Shoji M, Abe K. Induction of platelet derived-endothelial cell growth factor in the brain after ischemia. *Neurol Res* 2007;29:463–468. [PubMed: 17535565]
- Irvin DK, Dhaka A, Hicks C, Weinmaster G, Kornblum HI. Extrinsic and intrinsic factors governing cell fate in cortical progenitor cultures. *Dev Neurosci* 2003;25:162–172. [PubMed: 12966214]
- Justicia C, Perez-Asensio FJ, Burguete MC, Salom JB, Planas AM. Administration of transforming growth factor-alpha reduces infarct volume after transient focal cerebral ischemia in the rat. *J Cereb Blood Flow Metab* 2001;21:1097–1104. [PubMed: 11524614]
- Justicia C, Planas AM. Transforming growth factor-alpha acting at the epidermal growth factor receptor reduces infarct volume after permanent middle cerebral artery occlusion in rats. *J Cereb Blood Flow Metab* 1999;19:128–132. [PubMed: 10027766]
- Kaur B, Khwaja FW, Severson EA, Matheny SL, Brat DJ, Van Meir EG. Hypoxia and the hypoxia-inducible-factor pathway in glioma growth and angiogenesis. *Neuro Oncol* 2005;7:134–153. [PubMed: 15831232]
- Kornblum HI, Hussain RJ, Bronstein JM, Gall CM, Lee DC, Seroogy KB. Prenatal ontogeny of the epidermal growth factor receptor and its ligand, transforming growth factor alpha, in the rat brain. *J Comp Neurol* 1997;380:243–261. [PubMed: 9100135]
- Krupinski J, Kumar P, Kumar S, Kaluza J. Increased expression of TGF-beta 1 in brain tissue after ischemic stroke in humans. *Stroke* 1996;27:852–857. [PubMed: 8623105]
- Leker RR, Soldner F, Velasco I, Gavin DK, Androutsellis-Theotokis A, McKay RD. Long-lasting regeneration after ischemia in the cerebral cortex. *Stroke* 2007;38:153–161. [PubMed: 17122419]
- Leker RR, Teichner A, Grigoriadis N, Ovadia H, Brenneman DE, Fridkin M, Giladi E, Romano J, Gozes I. NAP, a femtomolar-acting peptide, protects the brain against ischemic injury by reducing apoptotic death. *Stroke* 2002;33:1085–1092. [PubMed: 11935065]
- Li Y, Lu Z, Keogh CL, Yu SP, Wei L. Erythropoietin-induced neurovascular protection, angiogenesis, and cerebral blood flow restoration after focal ischemia in mice. *J Cereb Blood Flow Metab* 2007;27:1043–1054. [PubMed: 17077815]
- Massengale M, Wagers AJ, Vogel H, Weissman IL. Hematopoietic cells maintain hematopoietic fates upon entering the brain. *J Exp Med* 2005;201:1579–1589. [PubMed: 15897275]

- Mezey E, Chandross KJ, Harta G, Maki RA, McKercher SR. Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. *Science* 2000;290:1779–1782. [PubMed: 11099419]
- Mezey E, Key S, Vogelsang G, Szalayova I, Lange GD, Crain B. Transplanted bone marrow generates new neurons in human brains. *Proc Natl Acad Sci U S A* 2003;100:1364–1369. [PubMed: 12538864]
- Monje ML, Toda H, Palmer TD. Inflammatory blockade restores adult hippocampal neurogenesis. *Science* 2003;302:1760–1765. [PubMed: 14615545]
- Ono K, Yoshihara K, Suzuki H, Tanaka KF, Takii T, Onozaki K, Sawada M. Preservation of hematopoietic properties in transplanted bone marrow cells in the brain. *J Neurosci Res* 2003;72:503–507. [PubMed: 12704811]
- Palmer TD, Willhoite AR, Gage FH. Vascular niche for adult hippocampal neurogenesis. *J Comp Neurol* 2000;425:479–494. [PubMed: 10975875]
- Sehara Y, Hayashi T, Deguchi K, Zhang H, Tsuchiya A, Yamashita T, Lukic V, Nagai M, Kamiya T, Abe K. Potentiation of neurogenesis and angiogenesis by G-CSF after focal cerebral ischemia in rats. *Brain Res* 2007;1151:142–149. [PubMed: 17459352]
- Six I, Gasan G, Mura E, Bordet R. Beneficial effect of pharmacological mobilization of bone marrow in experimental cerebral ischemia. *Eur J Pharmacol* 2003;458:327–328. [PubMed: 12504790]
- Swiercz JM, Kuner R, Behrens J, Offermanns S. Plexin-B1 directly interacts with PDZ-RhoGEF/LARG to regulate RhoA and growth cone morphology. *Neuron* 2002;35:51–63. [PubMed: 12123608]
- Thored P, Arvidsson A, Cacci E, Ahlenius H, Kallur T, Darsalia V, Ekdahl CT, Kokaia Z, Lindvall O. Persistent production of neurons from adult brain stem cells during recovery after stroke. *Stem Cells* 2006;24:739–747. [PubMed: 16210404]
- Toth ZE, Leker RR, Shahar T, Pastorino S, Szalayova I, Asemnew B, Key S, Parmelee A, Mayer B, Nemeth K, Bratinscak A, Mezey E. The combination of granulocyte colony-stimulating factor and stem cell factor significantly increases the number of bone marrow-derived endothelial cells in brains of mice following cerebral ischemia. *Blood* 2008;111:5544–5552. [PubMed: 18268092]
- Toth ZE, Mezey E. Simultaneous visualization of multiple antigens with tyramide signal amplification using antibodies from the same species. *J Histochem Cytochem* 2007;55:545–554. [PubMed: 17242468]
- Toth ZE, Shahar T, Leker R, Szalayova I, Bratinscak A, Key S, Lonyai A, Nemeth K, Mezey E. Sensitive detection of GFP utilizing tyramide signal amplification to overcome gene silencing. *Exp Cell Res* 2007;313:1943–1950. [PubMed: 17428464]
- Tsai JC, Goldman CK, Gillespie GY. Vascular endothelial growth factor in human glioma cell lines: induced secretion by EGF, PDGF-BB, and bFGF. *J Neurosurg* 1995;82:864–873. [PubMed: 7714613]
- Wang F, Zhu Y. The interaction of Nogo-66 receptor with Nogo-p4 inhibits the neuronal differentiation of neural stem cells. *Neuroscience* 2008;151:74–81. [PubMed: 18082332]
- Wang L, Zhang Z, Wang Y, Zhang R, Chopp M. Treatment of stroke with erythropoietin enhances neurogenesis and angiogenesis and improves neurological function in rats. *Stroke* 2004;35:1732–1737. [PubMed: 15178821]
- Willing AE, Vendrame M, Mallery J, Cassady CJ, Davis CD, Sanchez-Ramos J, Sanberg PR. Mobilized peripheral blood cells administered intravenously produce functional recovery in stroke. *Cell Transplant* 2003;12:449–454. [PubMed: 12911133]
- Zhang RL, LeTourneau Y, Gregg SR, Wang Y, Toh Y, Robin AM, Zhang ZG, Chopp M. Neuroblast division during migration toward the ischemic striatum: a study of dynamic migratory and proliferative characteristics of neuroblasts from the subventricular zone. *J Neurosci* 2007;27:3157–3162. [PubMed: 17376977]
- Zhang ZG, Zhang L, Croll SD, Chopp M. Angiopoietin-1 reduces cerebral blood vessel leakage and ischemic lesion volume after focal cerebral embolic ischemia in mice. *Neuroscience* 2002;113:683–687. [PubMed: 12150788]

Figure 1. Experimental design and time line

Timeline for the experiment is detailed. Please note that the day of stroke induction is day 0 and the day of 1st. irradiation therapy is day -30. The second irradiation dose was given also on day -30 but 6 hours after the first dose. Animals were kept in sterile environment for the first 10 days after irradiation and bone marrow transplantation and for another 20 days before stroke induction in normal non-sterile environment. Alzet mini-osmotic pumps containing TGF α or vehicle were inserted on the day of stroke induction and removed 14 days later. The animals survived for 90 days following stroke and were then killed and their brains were perfusion fixed for immunohistochemical evaluations.

Figure 2. TGF α increases GFP+ cell entrance into the ischemic brain

A. High power ($\times 400$) photomicrographs showing GFP+ (green) Y+ (red) cells in the ischemic hemisphere taken from an animal treated with TGF α . (panel a) or vehicle (panel b). Photographs were taken at the level of bregma +0.2 at the cortical border.

B. Bar graph chart depicting actual numbers of GFP+ cells in the ischemic and normal hemispheres in treated and control groups (n=10/group).

Figure 3. Treatment effects on blood vessels in the ischemic border zone

A. Medium power ($\times 200$) photomicrographs showing blood vessels stained with an antibody against von-Willebrand factor (green) from vehicle (a) or TGF α (b). Sections were taken from homologous areas surrounding the infarct cavity at bregma $- 0.2$.

Bars- 50 microns

B. Photomicrograph from the infarct border of an animal treated with TGF α showing that many of the GFP+ cells (green) localize to blood vessels. Some of these cells are BrdU+ (red; blue arrows). Many of the BrdU+ cells do not express GFP (yellow arrow). Nuclei are counterstained with DAPI (blue). Bar – 50 microns

C. Bar graph showing the area covered by blood vessels in the ischemic border zone in the different treatment groups (n=10/group).

Figure 4. Treatment effects on cell proliferation in the ischemic brain

A. Representative low power photomicrographs ($\times 100$) showing BrdU+ cells (green) in the ischemic hemisphere, from vehicle treated (a, c) and TGF α treated (b,d) animals. Panels a and b were taken from the non-ischemic hemisphere and panels b and d from the ischemic hemisphere. Light blue arrows in b and d point in the direction of the SVZ, which is out of frame in this magnification.

Bars – 50 microns

B. Bar graph depicting total numbers of BrdU+ cells in the ischemic SVZ and in the ischemic peri-infarct area from animals from different treatment groups (n=10/group).

A

B

* - $P < 0.05$ vs. Vehicle

Figure 5. Differentiation of newborn cells in the ischemic hemisphere

Z sections taken from animals treated with TGF α showing differentiation of BrdU+ cells (green) into NeuN expressing neurons (red a) away from the infarct border zone, GFAP expressing astrocytes at the infarct border zone (red b) and nestin expressing neural progenitors at the SVZ (red c). Sections were taken at bregma + 0.2 level. Green arrows point to double positive cells, and short blue and yellow arrows point to single positive cells expressing either BrdU (light blue arrows) or cell specific antigens (yellow arrows).

LV – lateral ventricle

bars – 50 microns

B. Bar graph showing differentiation of BrdU+ cells into fate specific cells in the different treatment groups (n=10/group)

Figure 6. Effects of TGF α on migrating neuroblasts in the ischemic hemisphere

A. High power ($\times 400$) photomicrograph from the brain of animal treated with intra-parenchymal TGF α killed 90 days after PMCAO. Sections taken at the level of bregma +0.8 show doublecortin (DCX, green) positive cells at the SVZ (a) and migrating away in chains towards the infarct (b). Note that DCX+ cells cluster together at the SVZ (red arrow in a) and migrate out of the SVZ as chains (blue arrow in a, and Figure b). Light blue and yellow arrows in b point in the direction of the lateral ventricle and the infarct core respectively. Many of these cells migrate along blood vessels (VWF+ red in c). Nuclei are counterstained with DAPI (blue).

LV – lateral ventricle, SVZ – subventricular zone

Bars 50 microns in a, b and 100 microns in c

B. Bar graph showing DCX+ cell numbers in the different treatment groups (n=10/group).

* - $P < 0.05$ vs. Vehicle

Figure 7. TGF α induces neuroprotection after stroke

Bar graph showing infarct volumes measured on brain section at 90 days after stroke (n=10/group). Results are shown as percentage of normal hemispheric volume to control for brain atrophy. Difference between TGF α and vehicle treated animals is statistically significant.

Table 1
Antibodies used for Immunohistochemistry

Antibody	Source	Host	Dilution	Pretreat	Incubation	Detection
GFP	Invitrogen	Rb	1:40000		1h at RT	a -Rb-HRP, FITC-Tyr
GFP	Chemicon	Chk	1:5000		1h at RT	a -Chk-Biotin, ABC,FITC-Tyr
GFP	Abcam	Rb	1:10000		1h at RT	a -Rb-HRP, FITC-Tyr
vWF	Vector	Rb	1:500	MW	O/N at 4°C	a -Rb-HRP, A594-Tyr
CD31	Abcam	Rb	1:200		O/N at 4°C	a -Rat A594 a -Rat A647
Iba1	WAKO	Rb	1:1000		1h at RT	a -Rb-HRP, A594-Tyr
BrdU	Accurate	Rat	1:200	MW	O/N at 4°C	a -Rat-HRP, A350-Tyr, a-Rat A488
DCX	Santa Cruz	Goat	1:200		O/N at 4°C	a -Goat A488
NESTIN	Gift from R. McKay	Rb	1:5000		O/N at 4°C	a -Rb A555
GFAP	DAKO	Rb	1:200		O/N at 4°C	a -Rb A555
NeuN	Chemicon	M	1:100		O/N at 4°C	a - M A555