Survivin-Dependent Angiogenesis in Ischemic Brain

Molecular Mechanisms of Hypoxia-Induced Up-Regulation

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Approaches to regulating angiogenesis in the brain, which may diminish parenchymal damage after stroke, are lacking. Survivin, the inhibitor of apoptosis protein, is up-regulated *in vitro* in vascular endothelial cells by angiogenic factors, including vascular endothelial cell growth factor (VEGF). To evaluate the *in vivo* role of survivin in the brain in response to hypoxia/ischemia, we used a mouse model of stroke and show that 2 days after permanent middle cerebral artery occlusion, survivin is uniquely expressed by microvessels that form in the peri-infarct and infarct regions. The extent of vascularization of the infarct is dependent on expression of survivin, since vessel density is significantly reduced in mice with heterozygous deficiency of the *survivin* gene (*survivin*^{+/-} mice), even though infarct sizes were not different. Hypoxia alone induces survivin expression in the brain, by cultured endothelial cells and by embryonic stem cells, but this response is at least partially independent of VEGF, hypoxia inducible factor 1 α , or placental growth factor. Delineating the spatiotemporal pattern of expression of survivin after stroke, and the molecular mechanisms by which this is regulated, may provide novel approaches to therapeutically optimize angiogenesis in a variety of ischemic disorders. (*Am J Pathol* 2003, 163:935–946)

Following acute cerebral ischemia due to diminished blood supply to the brain, deprivation of oxygen and glucose results in a series of biochemical events, leading eventually to cell death and often devastating functional neurological disturbances. Observations that postischemic neovascularization in the infarct and peri-infarct regions correlated with survival in patients with stroke¹ strongly suggested that angiogenesis might be a compensatory protective mechanism, and thus a potential therapeutic target. Therefore, major efforts are being

made to delineate the molecular events that regulate angiogenesis in the setting of stroke.

In rodent stroke models, neovascularization is evident within 1 to 3 days after middle cerebral artery occlusion (MCAO).² This occurs concomitantly with increased expression, by neurons, microglial cells, astrocytes and vessels, of the angiogenic molecule, vascular endothelial growth factor (VEGF).^{3,4} VEGF is the most prominent of the angiogenic factors, exerting its effects predominantly via VEGF tyrosine kinase receptors, VEGFR-1 and VEGFR-2, and neuropilins-1 and -2 (NP-1 and NP-2) (for review⁵). VEGF augments vascular endothelial cell growth and migration, but is also a potent vascular permeability factor. It further interferes with vascular endothelial apoptosis by promoting expression of inhibitors of apoptosis, including survivin^{6,7} and other cell-survival proteins such as Akt.⁸ While minimally expressed in normal adult brain,⁹ VEGF rapidly accumulates after ischemia, appearing in the peri-infarct and infarct regions¹⁰ and persisting for at least 7 days.¹¹ The postischemia neovascular response, driven largely by hypoxia-induced up-regulation and stabilization of VEGF, is initiated and maintained at least in part via hypoxia-inducible transcription factors, HIF1 α and HIF2 α .² In concert with enhanced expression of VEGF and its receptors, other growth factors and cellular receptors, including placental growth factor (Plgf), angiopoietin-1 and angiopoietin-2, tie-1 and tie-2,^{12,13} basic fibroblast growth factor (bFGF), thrombospondin-1 and thrombospondin-2,¹⁴ are also differentially regulated in highly specific spatio-temporal expression patterns, orchestrated to acutely minimize parenchymal damage, and to optimize subsequent healing and recovery (for review see^{9,15}).

Armed with these new insights, angiogenic agents are currently being evaluated for treating stroke. In this regard, a major focus has been directed toward testing the efficacy of VEGF. Administration of VEGF to rats 48 hours after middle cerebral artery (MCA) ischemia yielded beneficial effects with improved neurological

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recovery associated with enhanced angiogenesis.¹⁶ Furthermore, in a transient model of MCAO in rats, continuous infusion of VEGF into the lateral ventricle reduced infarct volume and cerebral edema, and appeared to have a direct neuroprotective effect.¹⁷ Nitric oxide, administered 24 hours after cerebral artery occlusion in rats, also enhanced angiogenesis in the ischemic brain, a response that was in part mediated by VEGF.¹⁸ In contrast to these promising findings, however, administration of VEGF after 1 hour of ischemia was complicated by blood brain barrier (BBB) leakage, hemorrhage, and larger ischemic lesions.¹⁶ This undesirable outcome, believed to result from vascular permeability-inducing properties of VEGF, was not observed when VEGF was administered directly and somewhat later to the surface of the postischemic brain.¹⁹ The early injurious effect of VEGF likely reflected relatively low-level expression of vasculo-protective molecules, such as angiopoietin-1, that at later postischemia time points are increased and protect the stability of the vessels.^{9,13} Thus, administration of angiopoietin-1, believed to enhance vascular integrity, plus VEGF, provided some protection against VEGF-induced BBB leakage shortly after focal ischemia.²⁰ Despite these advances, major gains in terms of stroke management have yet to be seen in the clinic. Further research is required to delineate the molecular mechanisms by which angiogenesis proceeds in the postischemic brain, so that a stable vascular network can be rapidly provided to rescue neurons from apoptosis/necrosis and to promote healing.

As noted, VEGF also promotes vascular endothelial cell survival by interfering with apoptosis. *In vitro* and *in vivo* studies have established that regulation of apoptotic pathways may impact stroke outcome.²¹ In models of cerebral ischemia, inhibition or gene-inactivation of caspase-3 or caspase-1 results in mice that are partially resistant to ischemia,^{22,23} while gene transfer of inhibitor of apoptosis proteins (IAPs) in normal rats also attenuates ischemic damage.²⁴ Survivin is a member of the IAP family, containing a single baculovirus IAP repeat (BIR) that facilitates interactions with caspase-3, caspase-7, and caspase-9.²⁵ Survivin has additional unique cell-survival properties, in that it is crucial for mitosis and cell-cycle progression.^{26–30} Notably, survivin is up-regulated in cultured vascular endothelial cells in response to angiogenic growth factors, including VEGF, angiopoietin-1, bFGF, and Plgf.^{6,7,31–33} Furthermore, while survivin expression is detected in several organs during development, it is prominent in the fetal brain,³⁴ maintaining expression into adulthood, where it is present in the choroid plexus, ependymal cells, neurons, astrocytes, and oligodendrocytes.³⁵

In view of the unique cell survival properties of survivin, its expression in ischemia-sensitive regions of the brain, and most notably its differential regulation in endothelial cells in response to VEGF and other angiogenic factors, we used a mouse model of stroke to test the hypothesis that survivin plays a crucial role in neovascularization and repair after cerebrovascular occlusion.

Materials and Methods

Transgenic Mice

Generation of *survivin*+/- mice by homologous recombination in embryonic stem (ES) cells has been previously reported.³⁶ For the current studies, transgenic mice were bred onto a Swiss:129s (~94:6) background. These animals express ~50% levels of *survivin* mRNA, and respond with increased sensitivity to FasL-induced hepatic apoptosis. No other phenotypic abnormalities have been identified. *Survivin*+/+ littermates were used as controls for experiments on *survivin*+/- mice. In other experiments to evaluate temporal changes in response to stroke, BALB/c mice were used.

Focal Cerebral Ischemia

Focal cerebral ischemia was achieved by permanent occlusion of the MCA according to established methods.³⁷ Briefly, 10- to 12-week-old mice weighing 20 to 30 g were anesthetized by intraperitoneal injection of ketamine and xylazine. Atropine (1 mg/kg) was administered intramuscularly, and body temperature was maintained at 37°C. A U-shaped incision was made between the left ear and left eye. The cranial and dorsal segments of the temporalis muscle were transected and retracted, thereby exposing the skull. A 2-mm-diameter opening was made in the region over the MCA with a handheld drill, with saline superfusion to prevent heat injury. The meninges were removed with a forceps, and the MCA was occluded with three adjacent ligations with 10–0 nylon thread (Ethicon, Johnson and Johnson, Dilbeek, Belgium). Finally, the artery was transected distal to the ligation. The temporalis muscle and skin were reconstructed, and the mice were allowed to recover. Control (sham) mice were treated identically, excluding ligation of the MCA. Mortality during the procedure did not exceed 10%. At different times (6 hours to 7 days) after MCAO, the mice were re-anesthetized and the brains were quickly removed and either fixed in 4% paraformaldehyde for 3 hours at 4°C and embedded in paraffin for subsequent histological analyses, or the intact brain was cut into slices and immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) in saline for quantitation of infarct volume (see below). In each case, a minimum of 4 to 7 mice was used at each interval.

Immunohistochemical and Vessel Density Studies

For histological analyses, 7- μ m brain sections were cut. Sections were dewaxed, rehydrated, antigen retrieval was performed, and they were incubated with proteinase K (20 mg/ml Tris-HCl, pH7.5) for 30 minutes at 37°C. Immunoperoxidase staining was performed according to standard techniques using the following antibodies: Specific rabbit anti-murine survivin antibodies were generated as described.³⁸ Rabbit anti-rat thrombomodulin (anti-TM) antibodies were a gift from Dr. R.W. Jackman

(Harvard University, Boston, MA). All other antibodies were obtained from SanverTECH (Boechout, Belgium). Vessel density was quantified by visually counting TM-staining vessels in 5 non-adjacent ($>100\ \mu\text{m}$ distant) cross-sections per mouse ($n = 4$) in at least 4 high-power fields per section,¹³ and dividing by the measured areas. Results are expressed as number of vessels/ mm^2 . The selected areas encompassed the entire penumbra and central infarct region. The leptomeninges, which is usually a site of increased vascularity postischemia, was excluded because the entire leptomeninges was not uniformly intact in all specimens after processing for immunohistologic analyses. All assessments were performed by one blinded microscopist. The mean value from each mouse was used to calculate the overall mean $\pm$ SEM.

Immunofluorescent Studies

Paraffin-embedded brain sections were processed as above, and co-incubated with primary endothelial cell-specific goat anti-Glut1³⁹ antibodies (Santa Cruz Biotechnology, SanverTECH) and biotinylated rabbit anti-survivin antibodies, or combinations of the corresponding pre-immune antibodies. After washes, sections were further incubated with rhodamine-conjugated anti-goat antibodies and FITC-conjugated streptavidin. Analyses were performed using a confocal laser-scanning microscope (Leica, Wetzlar, Germany). Settings to exclude non-specific background were determined by using the corresponding primary pre-immune antibodies.

Infarct Volume Measurement

After surgical removal of the brain and immersion of 1-mm slices in TTC, the stained sections were photographed, and the well-defined necrotic and apparently viable areas were quantified by planimetry, after which the infarct volume was calculated.⁴⁰ The intact contralateral hemispheric volume was also determined, and there was $\sim 2.5\%$ intermouse variability within the same strain of mice. Thus, absolute infarct volumes were used for the purposes of comparison.

Quantification of mRNA levels

Total RNA was isolated from homogenized tissue in TRIzol reagent (Life Technologies, Merelbeke, Belgium) according to the manufacturer's instructions. Standard curves for quantitative real-time polymerase chain reaction (PCR) were generated from plasmids containing cDNAs encoding murine survivin₁₄₀, VEGF, Plgf, HIF1 α , HIF2 α , and p53. Concurrent PCR to detect expression of the reference gene *HPRT*, using primers HPRTF (sense 5' ttatcagactgaagagctactgtaatgac) and HPRTR (antisense 5' ttaccagtgtcaattatattcttcaacaac) and probe 5'-(JOE)-tgagagatcatctccaccaataactttatgtccc-(TAMRA)-3' allowed for relative quantitation of expression of gene transcripts as a function of *HPRT* copy number. Real-time PCR measurements were done in triplicate, with dupli-

cate or triplicate samples under the same condition. Entire experiments were performed a minimum of two times.

Western Immunoblot

Cells were washed and lysed on ice in a solution containing 0.5% Triton X-100, 50 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetate, 10 mmol/L Tris-HCl pH7.5 in the presence of protease inhibitors. 100 μg were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions and transferred to a nylon filter which was blocked with 3% bovine serum albumin in TBS with 0.01% Tween and incubated for 2 to 18 hours with the primary antibody. After washing and incubation of the filter with the appropriate secondary antibody conjugated to horseradish peroxidase, detection was accomplished using the enhanced chemiluminescence method (Amersham-Pharmacia, Freiburg, Denmark). Equivalent loading was confirmed by re-blotting the filters for detection of actin.

Hypoxia Treatment of Mice

A regulated hypoxia chamber, maintained at room temperature, was used to expose mice to an ambient oxygen tension of 5.5% overnight. Mice were immediately sacrificed for analyses subsequent to hypoxia. Oxygen tensions were continuously monitored during experiments, and there was less than 1% variation from the target in the recorded oxygen tension during any experiment.

Cell Culture

ES cells lacking both alleles for the genes encoding *VEGF* and *HIF1 α* , with corresponding wild-type control cells, were generated at the Center for Transgene Technology and Gene Therapy by high G418 selection of ES cells heterozygous for each gene. ES cells were routinely cultured on fibroblast feeders and in the presence of leukemia inhibitory factor to prevent differentiation. Human umbilical vein endothelial cells (HUVECs) were cultured as previously described.⁴¹ Before hypoxia exposure, cells were split onto gelatin-coated plates, refed with complete media, and 24 hours later, incubated in a humidified hypoxia chamber for 20 hours before processing for RNA or Western blot analyses. An oxygen tension of 2% (5% CO₂, balance N₂) was selected due to reported effects on transcription of angiogenesis-related genes in endothelial cells under similar conditions.^{42,43} Studies were performed at least twice, with different ES cell clones, and real-time PCR was done in triplicate.

Animal Care

Animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Leuven.

Figure 1. Survivin expression in the brain. Expression of survivin was detected with specific anti-survivin antibodies visualized by immunoperoxidase staining in the hippocampus (**A** and **B**) and pyramidal cells in the cortex of the normal brains from BALB/c mice (**C**). There was no immunoperoxidase staining in the hippocampus (**D**) or in the cortex when pre-immune primary antibody was used. **E:** In a representative stroke 2 days after MCAO, H&E staining of a region within the frontal cortex reveals the area of infarct (**i**) above the dashed line in which nuclei are condensed and retracted (**arrows**), as compared with healthy-appearing cells (**arrowheads**) in the non-affected region below the line.

Statistical Analyses

Statistical analyses of data were conducted with the Stat-View computer program (Abacus Concepts Inc., Berkeley, CA) or with InStat 3 (MacKiev Company, Cupertino, CA). Student's *t*-test was used to compare groups in which the standard deviations were not significantly different. The data are represented as the mean $\pm$ SE.

Results

Survivin Expression in the Normal Brain

Using specific anti-survivin antibodies for immunoperoxidase staining of sections of fixed brain tissues, we first examined the expression pattern of survivin in the brains of normal adult BALB/c mice. Low levels of survivin were detected in neurons within the CA-1, CA-2, and CA-3 regions of the hippocampus, the dentate gyrus, and the pyramidal cells of the cortex (Figure 1, A to C), as similarly reported.³⁵ Survivin was not otherwise detectable in any other sites. Specifically, there was no expression in or around the vasculature. In adjacent sections, no signal was detectable when pre-immune Ig was used (Figure 1D), or when the primary antibody was excluded (not shown).

Survivin Expression by Brain Microvascular Endothelium after MCAO

Survivin expression in response to stroke was evaluated by using a murine model of permanent focal cerebral ischemia in which the MCA was ligated. Adult BALB/c mice were used because in pilot studies cerebral infarct volumes were consistently larger and there was less mouse-to-mouse variability than in other strains (eg, Swiss, 129s, C57Bl6). Demarcation of infarcts was readily identified by hematoxylin and eosin (H&E) staining (Figure 1E) as nuclei of cells within the infarct were condensed, and the cells retracted. From 6 hours to 7 days after MCAO, the expression of survivin remained detectable in both cerebral hemispheres in the neurons of the hippocampus, dentate gyrus, and cortex. The pattern and intensity of staining was similar to that seen in normal (untreated) or sham-treated (surgery, but without ligation of the MCA) mice. Only in the infarct region itself was survivin not identified in the neurons from 6 hours onwards. Furthermore, 6 hours post-MCAO (Figure 2A), survivin was also noted in the pia mater and pia vessels predominantly overlying the region of the infarct, while it was absent in corresponding sites in the contralateral, unaffected hemisphere. The same pattern and extent of expression was evident at 1 day post-MCAO (not shown). However, at 2 days post-MCAO prominent changes occurred. Survivin was detectable in what appeared morphologically to be microvessels within the peri-infarct and infarct regions (Figure 2, B and C), in a pattern suggestive of endothelial cell staining. Survivin expression was entirely absent in the vasculature of non-ischemic areas of the brain, the latter identified throughout the brain by endothelial-specific anti-TM antibodies. However, there was more intense survivin staining of pia mater and pia vessels in areas adjacent to and overlying the infarct (Figure 2C). Survivin was absent, however, in the corresponding regions of the contralateral hemisphere. This pattern of staining for survivin within the infarct was similar, although with slightly more prominent vascularity, at 3 days post-MCAO. Sections at all time points were stained in parallel with pre-immune primary antibody, and no signal was detected, thus excluding the possibility that the survivin-specific signals were artifactual (an example is shown in Figure 2H at 3 days post-PCAO). The similar pattern of staining in the peri-infarct and infarct regions with anti-TM antibodies provided additional support that survivin was being expressed by vascular endothelial cells (Figure 2D). Further confirmation, however, was accomplished by demonstrating co-localization of survivin to cells expressing the endothelial cell specific Glut1³⁹ (Figure 3). In the infarct region of the brain of mice 3 days post-MCAO (when vascular endothelial proliferation is reportedly maximal²), the patterns of staining for survivin and Glut1 (Figure 3A) were entirely overlapping. Non-specific signals from each labeled antibody were excluded by the use of corresponding preimmune antibodies in place of either anti-survivin antibody (Figure 3B.1) or anti-Glut1 antibody (Figure 3C.2). In the cortex of the contralateral hemisphere with respect to the

Figure 2. Expression of survivin and thrombomodulin in the brain post-MCAO. Representative sections of brains from BALB/c mice post-MCAO were immunostained for survivin or for thrombomodulin (TM). **A:** At 6 hours post-MCAO, survivin is most readily detected in the pia mater overlying the infarct region (arrows to the right of the dashed line) and in cortical pyramidal cells in the non-ischemic region (arrowheads to the left of the dashed line), while it is absent elsewhere. **B and C:** At 2 days post-MCAO, survivin staining appears in microvascular endothelial cells in the peri-infarct (**B**) and infarct (**C**) regions (arrowheads) and in the pia mater (arrows) and pia vessels. **D:** TM staining of microvascular endothelium is evident in the cortex of the brain 2 days post-MCAO, and is most prominent in the peri-infarct and infarct zone, which is to the right of the dashed line. The non-infarct region is to the left of the dashed line. The pattern of cell staining for TM in the infarct region (**F**, right of dashed line) is similar to that for survivin, as seen in **B** and **C**. **E to G:** At 7 days post-MCAO, brain sections were stained for survivin (**E** and **F**) and TM (**G**). The staining patterns of survivin and TM are similar in the penumbra (pen), pia mater and in the complex network of vascular endothelium (arrows) in the cortical infarct region. **H:** An example of the absence of non-specific immunoperoxidase staining of vascular endothelial cells (arrows) in the center of the cortical infarct region of the brain (3 days post-MCAO) when pre-immune antibodies were used.

stroke, Glut1 could be detected in vessels, while survivin was absent (Figure 3D). At 7 days post-MCAO, in concert with an increase in survivin and TM expression in the region of the infarct (Figure 2E to G), an irregular

Figure 3. Localization of survivin in cerebrovascular endothelium. Paraffin-embedded sections of brains from BALB/c mice 3 days post-MCAO were incubated with biotinylated-rabbit anti-survivin antibodies (**A.1**, **C.1**, **D.1**) and goat anti-Glut1 antibodies (**A.2**, **B.2**, **D.2**) or the appropriate pre-immune antibodies (**B.1**, **C.2**), and detected with FITC-conjugated streptavidin (left), and in the same microscopic fields, with rhodamine-conjugated anti-goat antibody (right), respectively. Positive staining is denoted with arrows. In the infarct region (**A** to **C**), endothelial cells are identified by staining with anti-Glut1 antibodies (**A.2** and **B.2**), and colocalize with survivin (**A.1**). FITC-labeled pre-immune antibodies exhibit no non-specific signal (**B.1**), nor do rhodamine-labeled pre-immune antibodies (**C.2**), in a region of the infarct where survivin is detected in endothelium (**C.1**). In the contralateral hemisphere (unaffected by the MCAO), Glut1 is readily detected in the vascular endothelium (**D.2**), while survivin is absent in the same field of view (**D.1**).

complex of vessels developed, extending apparently from the penumbra and the pia and leptomeninges overlying the infarct region, appearing to invade the core of the infarct.

Figure 4. Expression of VEGF in the brain post-MCAO. Representative sections of brains from BALB/c mice 0 hours (**A**), 6 hours (**B** and **C**), and 7 days (**D** and **E**) after MCAO were stained for VEGF. VEGF is normally present in cells of the hippocampus (CA1 region is shown) (**A**). VEGF expression is initially detectable 6 hours after MCAO in the pia overlying the infarct region (arrows in **B**), and in scattered glia-like cells in the infarct region of the cortex, occasionally adjacent to blood vessels (v) (**C**). At 7 days post-MCAO, VEGF staining (arrows in **D** and **E**) in the ipsilateral cortex is prominent in glia-like cells and evident near vessels (v) in the penumbra (pen), but less intensely in the central ischemic zone (i), and largely absent in the non-ischemic (ni) region (**E** is a high-power view of the dashed field in **D**).

Expression of VEGF post-MCAO

Since VEGF up-regulates survivin in vascular endothelial cells *in vitro*,^{6,32} we examined its expression pattern in the brains after stroke. Similar to previous reports,^{2,15} we detected low levels of VEGF in the hippocampus bilaterally in normal and sham-treated BALB/c mice (Figure 4A). At 6 hours post-MCAO, VEGF was also detected in the leptomeninges and pia overlying the infarct region (Figure 4B), and within the infarct in scattered glia-like cells, which were occasionally adjacent to vessels (Figure 4C). By 3 days post-MCAO, there was more VEGF staining by glia-like cells within the infarct region, particularly in the cortex, and close to vessels. The intensity of staining in the same spatial pattern was enhanced by 7 days post-MCAO, when VEGF expression was most prominent throughout the peri-infarct and leptomeningeal regions.

At this time, there was also somewhat less intense but readily identifiable VEGF expression within the core of the infarct, often adjacent to microvessels (Figure 4, D and E). Notably, from 3 to 7 days post-MCAO, the spatial pattern of expression of VEGF in and around the ischemic region was similar to that observed with survivin (Figure 2F), although the cellular source was clearly different.

Since VEGF and other angiogenic factors increase the expression of survivin *in vitro* in cultured endothelial cells via activation/phosphorylation of Akt,^{6,7,44} we also characterized the expression pattern of phosphorylated Akt (pAkt) in the brains in response to MCAO. Consistent with previous reports,⁴⁵ our studies revealed constitutive expression of phospho-Akt throughout the brain (not shown), most prominent in neurons, with a dramatic decrease in apparent expression within the infarct region by

Table 1. Infarct Volume Post-MCAO

Days post-MCAO	Infarct volume (mm ³ ± SEM)	
	<i>Survivin</i> ^{+/+} mice	<i>Survivin</i> ^{+/-} mice
1	18.2 ± 1.6 (<i>n</i> = 7)	15.9 ± 1.4 (<i>n</i> = 7)*
3	14.1 ± 2.0 (<i>n</i> = 7)	11.2 ± 0.8 (<i>n</i> = 8)*

**P* > 0.1.

After MCAO in transgenic mice, infarct volumes were determined by TTC staining as described in Materials and Methods. There were no significant differences between *survivin*^{+/+} and *survivin*^{+/-} mice.

6 hours post-MCAO. No specific staining of vascular endothelium for phospho-Akt was evident at any time during the 1 week following occlusion, suggesting that alternative mechanisms of up-regulation of endothelial cell survivin might be active.

Vascular Response after MCAO as a Function of Survivin Expression

In view of our findings that survivin expression by cerebrovascular endothelium is up-regulated in response to stroke, we predicted that vascularity and infarct size may be dependent on underlying levels of survivin. Total inactivation of the *survivin* gene in mice results in early embryonic lethality.^{30,36} *Survivin*^{+/-} mice appear phenotypically normal, yet under specific stresses, they exhibit enhanced sensitivity to proapoptotic stimuli.³⁶ To determine whether the response to cerebral hypoxia/ischemia would be altered by lower levels of survivin, we induced MCAO in adult and *survivin*^{+/-} mice and their sibling control wild-type *survivin*^{+/+} mice. At corresponding times post-MCAO, infarct volumes in *survivin*^{+/-} mice at 24 hours and 3 days were not significantly different from those of *survivin*^{+/+} mice (Table 1), suggesting that decreasing survivin expression by ~50% does not significantly alter infarct size in this permanent cerebral artery occlusion model. Since vessel formation in the ischemic region likely has an impact on recovery, we also quantified the density of vessels in the infarct region of the *survivin*^{+/+} and *survivin*^{+/-} mice at 3 days post-MCAO, before any evidence of volume retraction, and when endothelial proliferation is reportedly maximal in similar models in mice and rats.^{2,13} The density of TM-positive staining vessels within the penumbra and central infarct region (excluding the leptomeningeal area) was significantly diminished in the brains of *survivin*^{+/-} mice (508 ± 25 versus 404 ± 19 vessels/mm² in *survivin*^{+/+} and *survivin*^{+/-} mice, respectively (*P* = 0.003, *n* = 4 mice)). At 7 days post-MCAO, the vessel density was still greater in the strokes of the *survivin*^{+/+} mice (391 ± 26 vessels/mm²) as compared to those in *survivin*^{+/-} mice (299 ± 24 vessels/mm²), although the difference was no longer statistically significant (*P* = 0.068, *n* = 4 mice). The findings could not be attributed to differences in the numbers of vessels in the brain before MCAO; vessel densities in the corresponding regions of the cerebral cortex of untreated *survivin*^{+/+} mice (296 ± 21 vessels/mm²) and *survivin*^{+/-} mice (315 ± 32 vessels/mm²) were not significantly different (*P* > 0.1, *n* = 4). These

data are consistent with the notion that angiogenesis in response to cerebrovascular occlusion is partly dependent on survivin expression.

Oxygen-Dependent Regulation of Survivin Expression

Since hypoxia is a major angiogenic stimulus, we directly evaluated whether hypoxia contributed to the enhanced expression of survivin in the cerebrovasculature post-MCAO, by exposing wild-type *survivin*^{+/+} mice to ambient oxygen tensions of 5.5% for 20 hours. Survivin mRNA levels in the brains of mice exposed to hypoxia (*n* = 5) were 1.8 ± 0.4-fold higher than that seen in brains of mice under normoxic conditions (*n* = 8) (*P* < 0.05). The augmented expression of survivin was associated with an 8.6 ± 1.0-fold increase in VEGF mRNA levels in the brains of hypoxia-exposed mice, suggesting that VEGF could mediate the enhanced expression of survivin. Similar significant twofold and fivefold increases in survivin and VEGF mRNA levels, respectively, were obtained with BALB/c mice. To further explore the mechanism of hypoxia-induced up-regulation of survivin *in vitro*, we evaluated the expression of survivin by HUVECs following 20 hours of hypoxia. Under these conditions, survivin expression by endothelial cells reportedly increases in response to angiopoietin-1, bFGF, VEGF, and Plgf.^{6,7,33,44} In our studies, hypoxia resulted in a 2.1 ± 1.4-fold increase (*P* < 0.05, *n* = 3) in survivin mRNA levels (relative to normoxia), while VEGF and HIF1α mRNA accumulation was also enhanced (1.3 ± 0.2-fold and 1.5 ± 0.2-fold, respectively), but not to a level of significance (*P* > 0.05, *n* = 3). The data suggest that while VEGF likely plays a role in up-regulating survivin, there may be alternative VEGF-independent pathways by which hypoxia may induce survivin expression.

The regulation of survivin in response to hypoxia was therefore further studied by exposing undifferentiated *VEGF*^{-/-} and *VEGF*^{+/+} ES cells to hypoxia (2%) or normoxia for 20 hours (Figure 5A). Since inactivation of the *VEGF* gene results in embryonic lethality in mice,⁴⁶ the ES cell model provides mechanistic insights that otherwise are not available *in vivo*.⁴⁷ After 20 hours of exposure to hypoxia, survivin mRNA levels in *VEGF*^{+/+} ES cells were increased ~1.7-fold (*P* < 0.05). Expression of survivin protein was, however, not significantly affected, although monomeric forms appeared to diminish in intensity (Figure 5B). Under these conditions, VEGF mRNA levels increased by ~2-fold, concurrent with a significant 1.7-fold increase in accumulation of HIF1α mRNA (*P* < 0.05), and no change in HIF2α mRNA. The lack of more prominent changes in VEGF and HIF likely reflects the relatively mild degree of hypoxia used in these ES cell experiments as compared with other studies.^{47,48} As expected, VEGF mRNA was undetectable in the *VEGF*^{-/-} ES cells under all conditions. In the absence of VEGF, and associated with a significant increase in HIF1α mRNA levels, hypoxia enhanced survivin mRNA accumulation. Survivin protein expression was also increased, and appeared to more prominently affect accumulation of

Figure 5. Oxygen-dependent gene expression in *VEGF*^{-/-} ES cells. **A:** *VEGF*^{+/+} and *VEGF*^{-/-} ES cells were exposed to normoxia or hypoxia, and mRNA levels were quantified. Results reflect the mean $\pm$ SEM of experiments performed a minimum of three times. Asterisk indicates a significant increase ($P < 0.05$) relative to normoxia. **B:** After exposure of *VEGF*^{+/+} (+/+) and *VEGF*^{-/-} (-/-) ES cells to normoxia (20%) or hypoxia (2%), cells were washed with PBS and lysates were prepared. Western immunoblotting after SDS-PAGE on a 15% gel was performed with anti-survivin antibodies. Molecular weight markers are on the right. Survivin monomers, detected at ~16 kd appear to diminish after hypoxia, while dimers, recognized at ~32 kd, increased after hypoxia most prominently in *VEGF*^{-/-} ES cells. Equivalent loading was confirmed by immunoblotting stripped filters for actin (not shown).

the dimeric form (Figure 5B). While the data show that survivin may be up-regulated by hypoxia in the absence of VEGF, we could not exclude, from these experiments, a more direct role for HIF1 α in regulating survivin expression. We therefore examined the expression of survivin using *HIF1* α ^{-/-} and *HIF1* α ^{+/+} ES cells under normoxic and hypoxic conditions. In *HIF1* α ^{-/-} ES cells, HIF1 α mRNA was undetectable, while VEGF mRNA was only minimally expressed (Figure 6). The hypoxia-induced non-significant increase in VEGF mRNA may be attributable to stabilization of the mRNA, as opposed to a transcriptional effect of HIF1 α .⁴⁹ Survivin mRNA accumulation was significantly increased in the *HIF1* α ^{-/-} ES cells after hypoxia ($P < 0.05$), supporting the conclusion that survivin expression may, under specific conditions, be augmented in the absence of both VEGF and HIF1 α . The enhanced expression of survivin in the absence of HIF1 α suggests that HIF1 α may actually down-regulate survivin. Since HIF1 α can stabilize and/or up-regulate p53 during

Figure 6. Oxygen-dependent gene expression in *HIF1* α ^{-/-} ES cells. *HIF1* α ^{+/+} and *HIF1* α ^{-/-} ES cells were exposed to normoxia or hypoxia as indicated in the legend, and mRNA levels were quantified. Results reflect the mean $\pm$ SEM of experiments performed a minimum of three times. Asterisk indicates a significant increase ($P < 0.05$) relative to normoxia.

hypoxia,⁵⁰ and p53 may suppress survivin,⁵¹ we considered whether HIF1 α was acting on survivin via p53. However, the accumulation of p53 mRNA by the *VEGF*^{+/+} and *VEGF*^{-/-} ES cells (Figure 5A) was not affected under these experimental conditions, suggesting that p53 was not playing a critical role in regulating survivin expression in these cells. Since Plgf may also induce expression of survivin in endothelial cells,³³ we considered the possibility that hypoxia-induced up-regulation of survivin in the *HIF1* α ^{-/-} ES cells might be mediated by Plgf. However, this hypothesis was also excluded, as Plgf mRNA levels did not change in the ES cells in response to the hypoxia (Figure 6).

Discussion

Although few factors have been identified that enhance the expression of the IAP, survivin, these are predominantly and notably involved in angiogenesis. For example, treatment of cultured endothelial cells with angiotensin-1, bFGF, or VEGF augments survivin expression several-fold^{6,7} via activation of Akt and/or PI3 kinase pathways. Survivin expression by cultured human arteriolar endothelial cells is also enhanced in response to hypoxic preconditioning via PI3-kinase/Akt/NF- κ B signaling, which in turn facilitates tubular morphogenesis.⁵² Up-regulation of survivin may also confer protection to endothelial cells from stresses, including chemotherapy.⁵³ Despite these apparent links with angiogenesis, *in vivo* support for a direct role of survivin in vascular endothelial function, growth, and proliferation has been lack-

ing. We therefore used a mouse model of permanent focal cerebral ischemia to evaluate the role of survivin *in vivo*, and report the following: 1) survivin expression is augmented by cerebral capillary endothelial cells of vessels in the peri-infarct and infarct regions, and in the overlying pia mater following cerebrovascular occlusion, 2) the extent of vascularization in the first few days after cerebrovascular occlusion is dependent in part on survivin expression, and 3) survivin expression may be up-regulated in response to hypoxia by mechanisms not necessarily requiring VEGF, HIF1 α , or Plgf.

After cerebral ischemia due to vascular occlusion, the resultant diminished oxygen and nutrient delivery to the tissues leads to compensatory responses to protect the brain. One of the crucial mechanisms to enhance oxygenation is through new vessel formation. This may occur via sprouting of endothelial cells from pre-existing vessels, ie, angiogenesis (reviewed in⁵⁴), or may also involve vasculogenesis, whereby circulating endothelial progenitor cells from the bone marrow contribute to the newly forming vasculature in the ischemic region.⁵⁵ In rodent models of permanent MCAO, newly formed vessels are generally seen within 1 to 3 days in the peri-infarct region where cells are hypoxic.² Of the many factors involved in this response, VEGF is by far the best characterized. In most studies, VEGF mRNA is first detectable in the brain 3 to 6 hours after MCAO, mainly in glia-like cells and macrophages¹¹ in the penumbra. More sensitive detection methods suggest even a more immediate VEGF response.⁵⁶ VEGF protein expression peaks by ~2 days, when it is also found in the vicinity of capillaries within the core of the infarct¹⁵ and in the hippocampus.¹¹ Only after 2 to 3 days are VEGF and its receptors up-regulated within the ischemic zone, in the pia and leptomeninges,¹⁰ as vessels seem to "invade" the infarcted region, and vascular endothelial cells proliferate. Administration of VEGF in small animal stroke models has met with variable results, depending on the dose, time, and duration of administration.¹⁶ Shortly after an ischemic event, the capillary leakage induced by VEGF may be deleterious, whereas the angiogenic effect of VEGF may be beneficial for long-term recovery.¹⁶ That survivin and VEGF have overlapping temporal patterns of expression of their respective proteins, albeit by different cells, and that VEGF induces survivin expression *in vitro*, suggests that they may cooperate in tissue recovery efforts after hypoxic/ischemic injury. This is consistent with the current concept that VEGF does not act alone in enhancing the formation of competent new vessels after ischemia but rather functions in concert with finely tuned expression of an array of other angiogenic and anti-angiogenic regulators.

The roles and patterns of expression of other angiogenic molecules that notably also regulate survivin *in vitro*, have similarly been evaluated postischemia in rodent stroke models. For example, the expression of angiopoietin-1, an angiogenic factor that provides vessel stability, maintains vascular integrity, and induces sprouting, is variably reported to be acutely down-regulated in rodent brains post-MCAO,^{13,15} at a time when VEGF is initially increasing. It is interesting to note that constitutive

angiopoietin-1 expression in the adult brain is postulated to provide baseline protection against stresses that would disrupt the integrity of the BBB. It is possible that low levels of survivin, not detectable by immunostaining, play a role in this function. Angiopoietin-2, antagonistic to the vascular stabilizing influence of angiopoietin-1, starts to increase by 6 to 8 hours post-MCAO in endothelial cells within the infarct, peri-infarct, and hippocampus, persisting for up to 7 days. In concert with VEGF, angiopoietin-2 likely promotes vascular leakage early after the initial insult,¹⁵ allowing extravasation of macrophages from the circulation through the disrupted BBB, for subsequent "clean-up" of necrotic debris.⁵⁷ By 2 to 3 days post-MCAO, angiopoietin-1 is up-regulated, while BBB leakage is decreasing, and coincident with peak expression of both VEGF and survivin. The increased VEGF at this time does not induce leakage, likely due in part to the increasing presence of angiopoietin-1, which stabilizes the vessels. We speculate that survivin may enhance this stability. Endothelial cell proliferation is first noted at 24 hours and is preceded by a brief period of endothelial cell apoptosis (at ~12 hours) that appears to correlate with a decrease in VEGF/Ang2 ratio, and expression of Ang2 in those endothelial cells.¹³ Our finding that survivin expression is only apparent in the microvasculature of the infarct after ~2 days may reflect suppression of survivin's response by Ang-2, a hypothesis that has not yet been tested. However, this may prove to be clinically relevant, as interventions to increase survivin expression early after an hypoxic/ischemic cerebrovascular event might provide protection. Thus, it is critical to develop a thorough understanding of the molecular mechanisms that regulate survivin expression under these conditions.

While the preceding discussion implies that in the complex setting of cerebrovascular hypoxia/ischemia survivin is regulated *in vivo* by angiogenic factors, our studies indicate that alternative mechanisms may also be functional. There are numerous hypoxia-responsive genes, many of which are up-regulated following binding of HIF-1 α to hypoxia-responsive elements (reviewed in⁵⁸). These include, for example, VEGF, VEGFR-1, HIF1 α , and bFGF.⁵⁹ However, other pathways have been identified that are independent of HIF1 α , including NF κ B, early growth response factor-1, and metal transcription factors. Notably, HIF1 α has no role in increasing transcription of the gene encoding the inhibitor of apoptosis protein IAP-2 after hypoxia. A *cis*-acting cAMP response element-binding protein (CREB) site within the enhancer region of the IAP-2 gene appears to be critical for what is predominantly a transcriptional response.⁶⁰ Similarly, the induction of *Plgf* by hypoxia is dependent in part on a metal response element-binding transcription factor.⁶¹ We have not yet established which pathways are active *in vivo* in up-regulating survivin, or whether the response is mainly transcriptional or due to changes in mRNA stability. Indeed, it is likely that coordinate activation of one or more factors may be required for maximal expression of survivin under stress conditions. Overall, there is considerable evidence to support the existence of alternative hypoxia-responsive regulatory mechanisms that might be

amenable to therapeutic intervention, both in vascular hypoxia/ischemia and in tumor growth.

Surprising is our observation that the hypoxia-associated increase in survivin mRNA levels was more prominent in *VEGF*^{-/-} and *HIF1 α* ^{-/-} ES cells, as compared with the response in *VEGF*^{+/+} and *HIF1 α* ^{+/+} ES cells, respectively. These findings, confirmed in several ES cell clones under the same conditions, suggest that VEGF and/or HIF1 α suppress the response of survivin to hypoxia either directly or indirectly. Under hypoxic conditions, HIF1 α , in fact, modulates gene activity that may not only interfere with apoptosis, but under specific conditions, may also promote cell death.⁶² The molecular mechanisms that provide HIF1 α with pro-apoptotic properties are not yet fully delineated. Nonetheless, several responsible biochemical pathways have been identified. Most striking was the observation that HIF1 α stabilized p53 in tumors and promoted an apoptotic response.⁶³ This, however, does not appear to be the means by which survivin was up-regulated in our ES cells. HIF1 α has also been shown to induce endothelial cell cycle arrest and hypoxia-associated apoptosis by interfering with Bcl-2 expression,⁶⁴ and by up-regulating Nip3, a proapoptotic member of the Bcl-2 family.^{65,66} Further studies in other mammalian cell systems will be necessary to identify those factors that regulate survivin expression under hypoxic conditions.

Interestingly, in response to hypoxia, we found that the monomeric forms of survivin from ES cells decreased, while at least in the *VEGF*^{-/-} ES cells, expression of survivin dimers was enhanced. These findings are not entirely unique, since survivin dimers were similarly recognized in immunoblots of cultured human coronary vascular endothelial cells after exposure to hypoxia/reoxygenation.⁵² The crystal structure of survivin^{67–69} predicts that it forms dimers, which was postulated to have functional importance in either apoptosis and/or the cell cycle. Biochemical studies have confirmed that in the dimer conformation, survivin is effective at interfering with the activity of caspase-3 and caspase-7.⁷⁰ That changes in conformation might be a means of regulating functional expression of survivin, particularly as it pertains to angiogenesis in the setting of hypoxia, will be a topic for future investigations.

The early embryonic lethality of *survivin*^{-/-} embryos has confounded loss-of-function studies to more clearly define the role of survivin in vascular endothelial cell development and function, angiogenesis, and neurogenesis. Conditional, cell-specific inactivation studies in mice will provide the means to more directly answer these questions. These are in progress. Nonetheless, using a well-established mouse model representing stroke in humans, and complemented by *in vitro* studies, we have established that survivin is up-regulated in the microvasculature of the brain in response to cerebrovascular ischemia, and that the extent of vascularization within the infarct region is dependent in part on survivin. Identification of the mechanisms involved in regulation of the functional expression of survivin is crucial for the development of novel therapeutic approaches to treat and/or prevent not only stroke, but all illnesses in which the

outcome is dependent on vascular endothelial cell survival and death.

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