Ischemia/Reperfusion-Induced Neovascularization in the Cerebral Cortex of the Ovine Fetus

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Abstract

Information on the effects of injury on neovascularization in the immature brain is limited. We investigated the effects of ischemia on cerebral cortex neovascularization after the exposure of fetuses to 30 minutes of cerebral ischemia followed by 48 hours of reperfusion (I/R-48), 30 minutes of cerebral ischemia followed by 72 hours of reperfusion (I/R-72), or sham control treatment (Non-I/R). Immunohistochemical and morphometric analyses of cerebral cortex sections included immunostaining for glial fibrillary acidic protein and collagen type IV (a molecular component of the vascular basal lamina) to determine the glial vascular network in fetal brains and Ki67 as a proliferation marker. Cerebral cortices from I/R-48 and I/R-72 fetuses exhibited general responses to ischemia, including reactive astrocyte morphology, which was not observed in Non-I/R fetuses. Cell bodies of reactive proliferating astrocytes, along with large end-feet, surrounded the walls of cerebral cortex microvessels in addition to the thick collagen type IV–enriched basal lamina. Morphometric analysis of the Non-I/R group with the I/R-48 and I/R-72 groups revealed increased collagen type IV density in I/R-72 cerebral cortex microvessels (p < 0.01), which also frequently displayed a sprouting appearance characterized by growing tip cells and activated pericytes. Increases in cerebral cortex basic fibroblast growth factor were associated with neovascularization. We conclude that increased neovascularization in fetal cerebral cortices occurs within 72 hours of ischemia.

Key Words: Cerebral cortex, Fetus, Ischemia/reperfusion, Neovascularization, Ovine, Sheep

INTRODUCTION

Hypoxic-ischemic brain injury is the single most important neurologic problem occurring during the perinatal period. Substantial evidence suggests that a major component of brain injury is related to ischemia alone or hypoxia-ischemia (1, 2). In the brain, pathophysiologic responses to hypoxia-ischemia are highly complex and involve multiple mechanisms, including excitotoxicity, free radical damage, inflammation, and injury to the blood-brain barrier, all of which evolve in time and could predispose the brain to neuronal injury, glial injury, and cell death (3, 4). Considerable evidence also suggests that the onset of hypoxic-ischemic brain injury in the neonate may occur before birth (5–8).

The growth of new blood vessels from a preexisting vascular tree, known as angiogenesis, is a tightly controlled process that is regulated by angiogenic factors (9, 10). Angiogenesis is a multistep process that involves endothelial cells and pericytes and includes degradation of the existing vascular basement membrane with subsequent reassembly around newly formed blood vessels (11). During early ontogenesis in the human brain, blood vessels develop according to a specific pattern of angiogenesis (12); however, developmental remodeling of the vasculature to match local metabolic tissue demands is an ongoing process, and regulated angiogenesis is a crucial component of this process (11).

In addition to the angiogenesis that occurs during normal tissue development, neovascularization by angiogenic mechanisms is one of the events by which the vascular network can be increased in various pathologic situations, including wound healing, arthritis, cardiovascular diseases, cancer, and cerebral ischemia. Although the vascular system of the adult brain is principally stable under normal basal conditions, endothelial cells proliferate in response to brain ischemia (13). In recent years, accumulating information suggests that the damaged brain can be surprisingly plastic with regard to the coupling of angiogenic and neurogenic mechanisms (14). After ischemia, the production of nascent blood vessels facilitates neurorestorative processes, resulting in improved recovery (13). Endothelial cells in the ischemic boundary zone proliferate within 24 hours, resulting in active neovascularization within 3 days of traumatic brain injury (15). In adults, agents and manipulations that enhance neovascularization, neurogenesis, or both promote functional recovery after brain injury (15). These findings may be interpreted to suggest that the manipulation of and/or changes in endogenous neural precursors, endothelial cells, and endogenous/exogenous vascular growth factors that could augment these processes might represent potential therapeutic strategies for...
Studies in neonatal rodents suggest that angiogenesis is important for recovery from hypoxic insults in the brain (16). Increased density of proliferating brain capillaries was observed as early as Day 3 up to 2 weeks after hypoxia and ischemia (15, 16). Vascular endothelial growth factor (VEGF) is an important mediator of capillary proliferation; it is up-regulated after hypoxic-ischemic insults and is involved in angiogenesis, neurogenesis, and neuronal survival during the recovery process (17, 18). Disruption of the VEGF signaling pathway alters angiogenesis after recovery from hypoxic-ischemic brain injury in rodents (17, 19). In the adult brain, basic fibroblast growth factor (fibroblast growth factor-2 [FGF-2]) is another growth factor that has been shown to augment angiogenesis after ischemia (20, 21). Although numerous studies have characterized the neuronal response to a variety of ischemic insults in the fetal brain (22), there is very little information on the effects of ischemia/reperfusion-related injury on neovascularization in the cerebral cortex of the fetal brain (18, 23).

The ovine fetus has been used extensively to investigate many aspects of CNS development (24–28). We have previously shown that brain ischemia in the ovine fetus results in reproducible cerebral cortex and white matter lesions, increases in cerebral cortex FGF-2 levels, and increases in brain-blood barrier permeability, along with concomitant alterations in the molecular composition of endothelial cell tight junctions (4, 29). In addition, a recent study has shown that, at 48 hours after umbilical cord occlusion, the percentage of blood vessels expressing VEGF had increased in several brain regions (18). The rationale for the current study is that, although angiogenesis is an important component in fetal brain development (11, 30) and increases have been documented after ischemia and stroke in the adult brain (31), the effects of ischemia/reperfusion-related brain injury on the potential for neovascularization in the fetal brain are less well understood. In addition, the timing of the onset of neovascularization in the fetal brain after injury has not been well established. Knowledge of the responses of the immature vasculature to injury could be important for the development of therapies for hypoxic-ischemic injury in the immature brain (18, 23). Therefore, the objective of the current study was to test the hypothesis that ischemia/reperfusion induces neovascularization in the cerebral cortex of the ovine fetus. Furthermore, we also sought to examine the potential relative time course for the onset of neovascularization in the fetal brain after ischemia.

MATERIALS AND METHODS

This study was conducted with the approval of the institutional animal care and use committees of the Alpert Medical School of Brown University and Women & Infants Hospital of Rhode Island and in accordance with the National Institutes of Health guidelines for the use of experimental animals.

Animal Preparation, Study Groups, and Experimental Design

Surgery was performed on 12 mixed-breed pregnant ewes at 118 to 121 days of gestation under 1% to 2% isoflurane anesthesia, as described for our previous studies (29). Briefly, polyvinyl catheters were placed into a brachial artery and advanced to the thoracic aorta for blood sample withdrawal; they were then placed into a femoral artery and advanced into the thoracic aorta for heart rate and blood pressure monitoring, and into a brachial vein for drug administration (29). An amnionic fluid catheter was placed for pressure monitoring to correct fetal arterial pressure. Tissue samples for this study were obtained from animals in our previous report, which examined the effects of ischemia on white matter lesions in the fetus (29).

After exposure of the fetal carotid arteries, the vertebral-occipital anastomoses and lingual arteries were ligated to restrict vertebral and noncerebral blood flow, respectively (25). Two inflatable vascular occluders (In Vivo Metric, Healdsburg, CA) were placed around each carotid artery. For fetal electrocorticogram, 2 pairs of screws (Small Parts Inc, Miami Lakes, FL) were placed onto the dura, with a reference electrode sewn to the scalp (25, 29, 32). The screws were connected to a recorder by shielded polyvinyl chloride insulated wires (Alpha Wire Co, Elizabeth, NJ). The electrocorticogram was used to confirm isoelectricity during cerebral ischemia in the fetal sheep (29, 32). After recovery from surgery, at 123 to 129 days of gestation, the fetal sheep were assigned to 3 groups: 1) instrumented nonischemic sham control treatment (Non-I/R; n = 4); 2) ischemia induced by 30 minutes of carotid occlusion (hereafter designated as “ischemia”) followed by 48 hours of reperfusion (I/R-48; n = 4); and 3) ischemia followed by 72 hours of reperfusion (I/R-72; n = 4). At the conclusion of ischemia, the occluders were deflated and reperfusion was continued for 48 or 72 hours (29). In the Non-I/R group, the occluders were not inflated, but all other procedures were similar.

At the end of reperfusion, the ewe and fetus were killed with pentobarbital (100–200 mg/kg), hysterotomy was performed, and the fetus was withdrawn from the uterus. The brain was rapidly removed, and a portion of the anterior frontal cortex was obtained, immediately frozen in liquid N$_2$, and stored at −80°C until analysis for FGF-2. The remainder of the brain was placed in 10% formalin. Formalin-fixed whole brains were coronally cut at 1-cm intervals into 6 serial brain sections and paraffin embedded, as previously described (29). For this study, the paraffin-embedded anterior portion (i.e. Section 2) of the brain containing the frontal cortex (29) was used to determine collagen type IV (Coll IV), glial fibrillary acidic protein (GFAP), and Ki67 expression using immunofluorescence confocal microscopy.

Histology and Immunofluorescence Confocal Microscopy

Immunohistochemistry and morphometry were performed on the Non-I/R, I/R-48, and I/R-72 groups. Sections randomly chosen (n = 6 per brain) from the frontal cerebral cortex of the Non-I/R, I/R-48, and I/R-72 groups (n = 4 each) were processed for immunohistochemical and morphometric analyses as follows: Paraffin-embedded cerebral cortex samples were cut into 9-μm thick coronal sections (from the anterior horn of the lateral ventricles through the head of the caudate) and placed on Vectabond-treated slides (Vector Laboratories Inc, Burlingame, CA). Preservation of tissue structure and microanatomy of each brain section were ascertained by routinely staining sections with hematoxylin and eosin.
with toluidine blue at 300-μm intervals (n = 14 per brain). The sections were then dehydrated in acetone, clarified in xylene, and coverslipped with Entellan mounting medium (Merck, Darmstadt, Germany). Unstained sections were then selected for immunofluorescence, confocal analysis, and morphometry at intervals between toluidine-stained sections. This methodology guaranteed that only equivalent sections corresponding to the same cerebral cortex regions from each animal within the 3 groups were analyzed. Figures 1A–C show representative toluidine blue–stained sections from the Non-I/R, I/R-48, and I/R-72 groups and illustrate that the sections obtained from each group were similar.

Single immunostaining and double immunostaining were carried out with anti–Coll IV, anti-GFAP, and anti-Ki67 antibodies. Briefly, sections were rehydrated and processed for heat-mediated antigen retrieval by microwave pretreatment in 0.01 M citrate buffer (pH 6.0) for 15 minutes (3 × 5) at 750 W. The sections were then sequentially incubated with the following: 1) 0.5% Triton X-100 (Merck) in PBS for 30 minutes at room temperature; 2) blocking buffer (BB; PBS, 1% bovine serum albumin, and 2% fetal calf serum; Dako Italia, Milan, Italy) for 30 minutes at room temperature; 3) primary antibodies—mouse IgG1 anti-GFAP (diluted 1:100 in BB; Vision Biosystem Novoceastra, Newcastle upon Tyne, United Kingdom), rabbit anti–Coll IV (diluted 1:50 in BB; Abcam, Cambridge, United Kingdom), and Ki67 (diluted 1:50 in BB; Novus Biologicals, Littleton, CO)—overnight at 4°C; and 4) appropriate fluorochrome-conjugated secondary antibodies—goat anti-mouse IgG Alexa 555 (diluted 1:300 in BB; Invitrogen, Eugene, OR), biotinylated goat anti-rabbit (diluted 1:300 in BB; Vector Laboratories), and streptavidin-conjugated Alexa 488 (diluted 1:400 in BB; Invitrogen, Eugene, OR) for 30 minutes at room temperature; 5) at 750 W. The sections were then sequentially incubated with the following:

Morphometry and Quantitative Analysis
Quantitative evaluation of the percentage of Coll IV–stained area, as a measure of the vessel density of cerebral cortex microvessels, was performed by 2 independent observers (Francesco Girolamo and Marco Rizzi) who were not aware of the group designations, using computer-aided morphometry on the microscopic images. An average of 15 fields per section from each brain (n = 6 sections per n = 12 brains), for a total of 360 fields in each group (Non-I/R, n = 4; I/R-48, n = 4; I/R-72, n = 4; total cerebral cortex fields, n = 1,080), were analyzed by morphometry. Confocal images were optimized by contrast enhancement functions and digital filters and segmented by an interactive thresholding modality, and the resulting binary image was processed by the measurement function of ImageJ software (National Institutes of Health, Bethesda, MD). For each Coll IV immunolabeled field, images of the immunoreactive areas of each vessel were acquired at 20× (total area, 600.62 μm²) and measured on a single channel and z-stacks of 15 single optical planes.

FGF-2 Assay
The protein concentration of FGF-2 in the frontal cerebral cortex was measured by ELISA, as previously described (29). Briefly, frozen samples from the anterior frontal cortex were homogenized on ice in a buffer consisting of 20 mmol/L Tris-HCl (pH 7.4), 2.0 M NaCl, 1 mmol/L EDTA, 1 mmol/L ethylene glycol tetraacetic acid, 0.5% deoxycholate, 1% Igepal, and proteinase inhibitor cocktail (1 mmol/L phenylmethylsulfonyl fluoride and 1 μg/mL of each of the following: aprotinin, leupeptin, and pepstatin A). The samples were centrifuged at 14,000 rpm for 30 minutes at 4°C, and the total protein content of clarified supernatants was determined with a commercially available assay kit (Pierce, Rockford, IL). The FGF-2 content of the supernatants was determined by ELISA using a human FGF-2 immunoassay (Quantikine; R&D Systems, Minneapolis, MN). The assay procedures were performed according to the

**FIGURE 1.** Representative toluidine blue–stained sections from the Non-I/R (A), I/R-48 (B), and I/R-72 (C) groups taken at regular intervals and used to identify similar brain levels and cerebral cortex areas for morphometric analysis. Scale bars = 5 mm. Non-I/R, sham control treatment; I/R-48, ischemia/reperfusion for 48 hours; I/R-72, ischemia/reperfusion for 72 hours.
manufacturer’s specifications. An ELISA scanner (Titertek Multiscan Plus MKII; ICN, Costa Mesa, CA) was used to measure the optical density of total protein at 562 nm and the optical density of FGF-2 at 450 nm. Values for FGF-2 and quantitative evaluation of the percentage of Coll IV-stained vessel area were available for the Non-I/R (n = 3), I/R-48 (n = 3), and I/R-72 (n = 2) groups.

**Statistical Analysis**

Results from each group were expressed as percentage area (mean ± SD). All immunohistochemical data were statistically analyzed using 1-way analysis of variance and Bonferroni multiple comparison test (GraphPad Prism; GraphPad Software Inc, La Jolla, CA). Correlational analysis also was used to compare the percentage of Coll IV–stained vessels within the same animals and using a least-squares computerized linear regression program (StatSoft, Tulsa, OK). Results were considered significant at p values of 0.05 or less.

**RESULTS**

The fetal sheep from which the cerebral cortex samples were obtained had pH, arterial blood gas, heart rate, and mean arterial blood pressure values within the physiologic ranges within each group (29); similar values have been reported in other laboratories (33).

**Immunofluorescence Confocal Microscopy, Morphometry, and Statistical Analysis**

Double immunolabeling was performed with anti-GFAP antibodies as a marker of astrocytes and anti–Coll IV antibodies as a molecular component of the vascular basal lamina (VBL).
to reveal the vascular network profiles in the cerebral cortices in the Non-I/R, I/R-48, and I/R-72 groups. Compared with the cerebral cortex in the Non-I/R group, the cerebral cortices of the fetuses at both 48 and 72 hours after ischemia showed evidence of a generalized response to ischemic injury throughout the cerebral cortex areas (Fig. 2). Ischemia/reperfusion in the cerebral cortex induced reactive-type astrocyte morphology and a proliferative response in these cells, consistent with our previous report (29). In the Non-I/R group, the bodies and processes of GFAP-reactive astrocytes belonging to the glia limitans abutted the pial surface and tightly enveloped the penetrating cerebral cortex microvessels (Fig. 2A). Throughout the cerebral cortex layers, GFAP-reactive protoplasmic astrocytes showed fine morphology and formed typical perivascular end-feet that had extensive contact with the surface of a number of small cortical microvessels that were revealed by their detectable Coll IV VBL (Figs. 2A, B).

In the I/R-48 and I/R-72 groups, the glia limitans, parenchymal astrocytes, and perivascular astrocytes all responded to the ischemia/reperfusion experimental conditions by hypertrophy, swelling, rapidly upregulated GFAP immunoreactivity, and possibly proliferated (i.e. reactive) astrogliosis. Hypertrophic astrocytes showed a large body and a high number of processes throughout all the cerebral cortex layers (Figs. 2C–F). Numerous bodies and processes extensively covered the pial surface and formed a dense network in the entire neuropil (Figs. 2C–F). Like their normal counterparts, reactive astrocytes contributed large perivascular end-feet to microvessel walls (Figs. 3A–F), the profiles of which were revealed by the presence of a continuous, thick, Coll IV–enriched VBL (Figs. 3C–F). In particular, in the I/R-72 cerebral cortex, Coll IV immunolabeling revealed a number of small seamless-like microvessels suggestive of activated vessel proliferation by angiogenic mechanisms (11) (Figs. 2E, F, 3E, F). These aspects of increased vessel density prompted a detailed analysis of the cerebral cortex vascular network to ascertain the occurrence of neovessel formation. Experimental animals (I/R-48 and I/R-72) were compared with Non-I/R animals by applying an interactive morphometric procedure on single-channel (Coll IV; green)/binary confocal images (Figs. 4A–C). To measure the percentage of Coll IV–labeled

**FIGURE 3.** Confocal images of sheep fetal cerebral cortex double-immunostained for GFAP and Coll IV. (A–F) Compared with Non-I/R cerebral cortices (A, B), diffuse astrogliosis is clearly recognizable in both I/R-48 (C, D) and I/R-72 (E, F) groups; hypertrophic astrocytes form perivascular end-feet in contact with a thick Coll IV–enriched VBL (arrowheads in C–F). Nuclear counterstaining, TO-PRO3. Scale bars = (A, C, E) 25 μm; (B, D, F) 10 μm. Non-I/R, sham control treatment; I/R-48, ischemia/reperfusion for 48 hours; I/R-72, ischemia/reperfusion for 72 hours.

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areas, we calculated the “mean area fraction” on randomly analyzed cortex fields (total fields, n = 1,080). The results from each group were expressed as percentage area (mean \( \pm SD \)). The mean vessel density of the I/R-48 group did not show a significant difference from the mean vessel density of the instrumented Non-I/R group (I/R-48, 2.67% \( \pm 0.41% \); Non-I/R, 2.02% \( \pm 1.01% \)). By contrast, the I/R-72 group displayed a vessel density that was significantly higher than those of the Non-I/R group (p < 0.01, I/R-72, 5.11% \( \pm 1.54% \)) and the I/R-48 group (p < 0.05; Fig. 5).

These data were supported by observations carried out by single Coll IV immunostaining and double Coll IV/GFAP immunostaining at higher magnifications (Figs. 6A–F, 7A–D). In these cerebral cortex fields, sprouting-type microvessels (already present, though rare, in I/R-48 animals) (Figs. 6A, B) became a common feature of I/R-72 cerebral cortices (Figs. 6C–F). At this time, endothelial cell proliferation was recognizable as hypercellularity of the vessel wall, which also appeared to be surrounded by a number of pericytes that were typically enclosed by VBL (Fig. 7A). These growing vessels were characterized by endothelial tip cells and activated pericytes forming points of vessel sprouting and always surrounded by perivascular reactive astrocytes and a Coll IV VBL (Figs. 6A–F, 7B–D) (11, 34). In the cerebral cortices of the I/R-72 group, growing microvessels consistently exhibited a high density of perivascular hypertrophic astrocytes, in contact with a very thick Coll IV VBL, which characteristically ensheathed the entire associated pericytes (Figs. 8A–F). Emerging vascular tubes exhibiting a faint reactivity to Coll IV staining were frequent and consistently ensheathed by perivascular astrocytic end-feet (Figs. 8A, B, D, E). In areas of astrogliosis, the presence of several pairs of perivascular astrocytes suggested that ischemia/reperfusion resulted in reactive hyperplastic astrocytes (Fig. 8C).

The cell proliferation marker Ki67, along with GFAP and Coll IV, was examined by immunohistochemical double staining to determine the tendency of the I/R-72 cerebral cortex astrocytes to exhibit hypertrophic/hyperplastic changes and to determine the ability of the microvessels to grow by means of angiogenic mechanisms. The cerebral cortices from I/R-72 fetuses displayed numerous Ki67-reactive nuclei in both vascular and parenchymal cellular elements (Fig. 9) compared with the Non-I/R and I/R-48 groups, which showed extremely rare Ki67-immunostained nuclei (data not shown). Ki67-reactive endothelial nuclei were frequently identified in small blood vessels surrounded by hypertrophic, strongly GFAP-reactive, perivascular astroglia in the Ki67/GFAP-labeled cerebral cortices.
of the I/R-72 group (Figs. 9A–D). Growing microvessels were observed in close relationship to a large number of Ki67-labeled proliferating astrocytes (Figs. 9A–D). Doublets of intensely stained, hypertrophic, proliferating astrocytes stained with both GFAP and Ki67 were frequently observed at these sites. The findings of doublets of astrocytes strongly support the contention that there is a pool of astrocytes capable of entering the mitotic cell cycle and that proliferative events can be detected in the fetal brain 72 hours after ischemia.

Double staining for Coll IV and Ki67 identified proliferating endothelial cells, confirming that cerebral cortex microvessels were frequently characterized by mitotic events in I/R-72 animals (Figs. 9E, F). Variations in the intensity of Ki67 staining within the karyoplasm, ranging from weak to intense, also are consistent with the early and late mitotic phases of the cell cycle, respectively (Figs. 9A–F) (35).

Based on standard morphologic criteria, we investigated apoptosis-related cell deaths using light microscopy (original magnification: 1,000×) on toluidine blue–stained sections. After an initial survey, cells that showed shrinkage and nuclear condensation or fragmentation were considered identifiable as apoptotic cells but were only apparent in a few areas of the parenchyma in the frontal cortex. Moreover, apoptosis was not identified in the microvessels that had been exposed to ischemia/reperfusion for 48 or 72 hours.

Correlational Analysis of Neovascularization, Pathologic Scores, and FGF-2 Protein Concentration in the Frontal Cerebral Cortex

As previously reported, ischemia/reperfusion increases the pathologic injury scores of coronally sliced whole-brain sections stained with Luxol fast blue/hematoxylin and eosin (29). Ischemia for 30 minutes with reperfusion for 48 and 72 hours resulted in greater damage across 6 brain slices compared with brain slices from the sham-treated control group (29). In a previous work, nuclear pyknosis, cytoplasmic reddening, and hyperchromatism were considered indicative of neuronal injury (29). Evidence of ischemic damage was also apparent in the frontal cortex—the brain region where the percentages of Coll

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**FIGURE 6.** Confocal images of sheep fetal cerebral cortex double-immunostained for GFAP and Coll IV: merged (A, C, E) and single-channel (B, D, F) images. (A, B) A rare sprouting-like microvessel in the cerebral cortex from the I/R-48 group; the endothelial cell tip shows a long exploring filopodium surrounded by reactive astrocytes (arrowheads). (C–F) Sprouting-like vascular endings (arrowheads) surrounded by a number of seamless-appearing microvessels (asterisks) in the I/R-72 group. In (A) to (F), pericytes are included in the Coll IV–reactive VBL (arrows). Nuclear counterstaining, TO-PRO3. I/R-48, ischemia/reperfusion for 48 hours; I/R-72, ischemia/reperfusion for 72 hours. Scale bars = 10 µm.
The objectives of the current study were to determine whether ischemia/reperfusion induces neovascularization in the cerebral cortex of the ovine fetus and to establish the relative time course for the commencement of neovascularization in the fetal brain after injury. This study has 3 main findings. First, Coll IV–labeled reactive sprouting-type microvessels are apparent, though sparse, within 48 hours of ischemia. Second, these reactive sprouting-type microvessels become a common feature by 72 hours after ischemic brain injury in the fetus, suggesting that increased neovascularization in the fetal cerebral cortex occurs within 72 hours of ischemia. Third, increases in cerebral cortex FGF-2 concentration are associated with increases in neovascularization in the ovine cerebral cortex. Consequently, increased neovascularization is a component of ischemic injury in the fetal brain.

The neurodevelopment of the immature ovine brain is similar to that of the premature human infant brain with respect to completion of neurogenesis, onset of cerebral sulcation, and detection of evoked potentials (24, 36–38). Full-term pregnancy in sheep takes 145 to 148 days of gestation. We examined fetal sheep at 123 to 129 days of gestation, which represents approximately 85% of ovine gestation and therefore is at a time in gestation that is approximately similar to late preterm and near term in human infants (24). Although rodents are frequently used to study brain development and injury, the rodent brain is immature at birth (39) and almost completely agyric. In contrast, similar to nonhuman primate brain and the human brain, the sheep brain develops prenatally and is gyrencephalic. Therefore, studies of neovascularization after ischemia/reperfusion injury in the ovine fetus at 85% of gestation could have relevance to events occurring in near-term infants after brain injury (25, 29).

Neurogenesis and angiogenesis can occur in response to ischemic brain injury and could potentially be enhanced by processes that attenuate brain injury (40). In vitro evidence suggests that endothelial cells can secrete active substances that influence neuronal survival and thereby facilitate neuroprotective effects after ischemic insults (41). The immunohistochemical and morphometric analyses in the current study demonstrated that, compared with controls, the mean vessel density was significantly greater in the fetuses 72 hours after ischemia than 48 hours after ischemia and that a trend in microvessel activation was suggested by evidence of endothelial cell/pericyte activation and proliferation. These findings were confirmed by endothelial reactivity for the proliferative cellular marker Ki67 and by clearly recognizable sprouting-type microvessels. Consequently, cerebral neovascularization in the fetal cerebral cortex seems to occur within 72 hours of ischemia. The reactive astrocytes surrounding cerebral cortex microvessels after ischemia/reperfusion exhibited classical features of increased GFAP expression and cytoplasmic hypertrophy and increased proliferative activity. This elevated proliferative index was confirmed by the occurrence of Ki67-reactive astrocytic nuclei, together with the presence of Ki67 immunoreactivity, in doublet astrocytes. Similar changes in the proliferation of perivascular astrocytes have recently been described using live imaging in vivo after brain injury (42). Such studies suggest
that specific groups of perivascular astrocytes are involved in this process and that these cells may share similarities with perivascular astrocytic proliferation observed after a wound lesion in the brain (43). However, the effects of the observed early neovascularization and astrocytic proliferation on neurogenesis in the fetal brain remain to be determined.

Although very few studies have focused on the microvascular responses of the near-term fetal brain to injury (18, 23), several previous studies have suggested that the onset of angiogenesis begins within several days of ischemic brain injury both in adults and in neonatal rodents; these findings are based on results from a variety of models, including adult and neonatal stroke (14, 44), prolonged hypoxia (16), and hypoxia-ischemia (15). Angiogenesis-related genes, such as VEGF, were upregulated within minutes of focal cerebral ischemia in adult rodents, generating proteins that remain elevated in astrocytes in the ischemic area for days to weeks (14). In addition to the presence of increased VEGF, endothelial cell proliferation has been reported to occur within 12 to 24 hours of stroke and to continue for a number of weeks (14). Similarly, angiogenesis-related genes and proteins, including hypoxia-inducible factor-1α and VEGF, increase within hours of stroke in neonatal rodents (44). The number of hypoxia-inducible factor-1α–positive cells began to increase at 4 hours, peaked at 8 hours, and decreased at 24 hours after transiently occluding the middle cerebral artery for 1.5 hours in neonatal rats (44). Increases in VEGF messenger RNA and VEGF protein seem to correlate with angiogenesis in postnatally developing rat brains (16, 44). Vessel density also seemed to increase within 3 days of hypoxic-ischemic injury in neonatal rats (15). Therefore, our findings in the ovine fetus after ischemia/reperfusion, combined with previous findings in neonatal rodents, suggest that the onset of neovascularization can occur within 48 hours of injury both in fetuses and in neonates, and that neovascularization occurs after stroke (44) and hypoxia (16) in neonatal rodents and after ischemia/reperfusion brain injury in the fetus.

Our findings are consistent with a recent report showing significant increases in the percentage of blood vessels expressing VEGF in the subventricular zone, periventricular white matter, and subcortical white matter at 24 and 48 hours.
after umbilical cord occlusion in the ovine fetus (18). Umbilical cord occlusion has recently been shown to result in decreased vascular density in the caudate nucleus, along with a shift in the frequency of smaller- to larger-perimeter blood vessels in periventricular and subcortical white matter, whereas both blood vessel density and morphology remain stable up to 48 hours after ischemia in the cerebral cortex (23). These findings, combined with our results indicating an increased rate of cerebral cortex neovessel formation 72 hours after ischemia, reveal endothelial cell/pericyte activation and vascular sprout formation (e.g. endoglin/CD105 and proteoglycan NG2, respectively), as we have previously have reported for human angiogenesis (11, 45, 46). Consequently, more selective markers for detecting the onset of neovascularization could not be determined. Nonetheless, by immunostaining for Coll IV as an early component of the VBL, we were able to detect vascular sprouting after ischemia in the fetal sheep brain, which appeared similar to the vascular sprouting that we have previously reported during angiogenesis in the human fetal brain. In fact, immunofluorescence data demonstrated that deposition by endothelial cells of Coll IV in the VBL is a rather early event during angiogenesis and that discontinuities in the VBL at the tip of growing vessels may be smaller than previously thought (47). On this basis, although adequate markers were not available to localize vascular sprout formation in the ovine brain specifically, our
findings are consistent with previous work documenting endothelial/pericyte mobilization in actively growing vascular sprouts (12, 34, 45) and are consistent with similar conclusions in a recent report indicating that increased vascular VEGF expression was reported in several brain regions after fetal exposure to umbilical cord occlusion (18). Administration of this angiogenic factor into the adult rat brain also resulted in unanticipated proliferative effects on astroglia (48). Although endothelial cells do not represent the major source of VEGF, they do have the capacity to release this growth factor in microvessels (49) and thereby stimulate the marked proliferative responses that we observed in perivascular astrocytes. However, disruption or loss of basal lamina components has been reported to occur after ischemia, resulting in damage or loss of microvascular integrity (50–52). In contrast to these findings in adult rats, we observed an increased thickness of Coll IV–stained VBL in fetal sheep after ischemia. Therefore, discrepancies between previous findings in adult rats and our findings in fetal sheep should be considered in view of the different experimental models examined. First, there are differences in the duration of ischemia/reperfusion, brain regions analyzed, and microvasculature organization and features. Second, there could be differences in vascular reactivity between the fetal brain and the adult brain, particularly when considering aged animals. Third, there could be differences in the response of immature endothelial cells and pericytes to sudden or extreme shifts in cerebral cortex microcirculatory flow. Therefore, our present findings in the fetal brain after ischemia do not support the contention that there is a loss of basal lamina components, as previously reported in the adult rat (50–52). Additional ultrastructural analysis is required to delineate the dynamic relationships that take place between vascular cellular and noncellular components during ischemia/reperfusion in the developing cerebral cortex.

Fibroblast growth factor-2 has been proposed to induce angiogenesis after ischemia in the adult brain. Fibroblast growth factor-2 has been detected in neurons, astrocytes, and endothelial cells after ischemic stroke in adult patients (53). Its expression in ischemic areas is elevated compared with its expression in infarcted tissue and in the normal contralateral hemisphere (53). In a study of 30 patients with acute cerebral infarction, serum basic fibroblast growth factor levels increased significantly after infarction, peaking on Day 3 and persisting until Day 14, and showed a positive correlation between peak basic fibroblast growth factor levels and neurologic deficit improvements (54). Similarly, FGF-2 was found to sustain blood vessels in vitro and to maintain tight junction composition in neonatal mouse brain (55). The vascular architecture of the neonatal mouse brain was more complex, and the vessels appeared as continuous structures as early as Day 3, indicating a role for FGF-2 in angiogenesis (55). We previously reported that the concentration of FGF-2 increased in proportion to the severity of brain damage in the cerebral cortex of fetal sheep, peaking after 72 hours of reperfusion; we speculated that FGF-2 played a role in the attenuation of further injury in the fetus (29). The present study demonstrated a direct linear correlation between neovascularization as quantified by Coll IV expression and FGF-2 concentration, using our previously published FGF-2 measurements (29). The correlation between area fractional vessel density (in percentages) in the frontal cerebral cortex and FGF-2 concentration and Coll IV suggests that increases in FGF-2 are also associated with increased neovascularization. Our findings in the fetal brain are consistent with previous work suggesting that FGF-2 can induce angiogenesis after ischemia in the adult brain (55).

In conclusion, neovascularization occurs within 72 hours of the exposure of the fetal brain to ischemia/reperfusion. Increases in cerebral cortex FGF-2 concentration may be involved in this process. Our findings, taken together with recent work (4, 18, 23), suggest that perturbations in the microvasculature seem to represent an important component of hypoxic-ischemic injury to the fetal brain.

REFERENCES


