Effect of Prenatal Glucocorticoids on Cerebral Vasculature of the Developing Brain

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Background and Purpose—Prenatal glucocorticoids prevent germinal matrix hemorrhage in premature infants. The underlying mechanism, however, is elusive. Germinal matrix is enriched with angiogenic vessels exhibiting paucity of pericytes and glial fibrillary acidic protein-positive astrocyte end feet. Therefore, we asked whether glucocorticoid treatment would suppress angiogenesis and enhance periendothelial coverage by pericytes and glial fibrillary acidic protein-positive end feet in the germinal matrix microvasculature.

Methods—We treated pregnant rabbits with intramuscular betamethasone and delivered pups prematurely by cesarean section at E29 (term=32 days). Endothelial turnover, vascular density, pericyte coverage, glial fibrillary acidic protein-positive end feet, cell death, and growth factors orchestrating angiogenesis, including vascular endothelial growth factor, angiopoietins, transforming growth factor-β, and platelet-derived growth factor-B, were compared between betamethasone-treated and untreated pups. Similar comparisons were done between autopsy materials from premature infants exposed and unexposed to prenatal glucocorticoids.

Results—Antenatal glucocorticoid treatment reduced endothelial proliferation, vascular density, and vascular endothelial growth factor expression in the germinal matrix of both rabbits and humans. The pericyte coverage was greater in glucocorticoid-treated rabbit pups and human infants than in controls, but not the glial fibrillary acidic protein-positive end feet coverage. Transforming growth factor-β, but not angiopoietins and platelet-derived growth factor-B, were elevated in glucocorticoid-treated rabbit pups compared with controls. Betamethasone treatment induced apoptosis, neuronal degeneration, and gliosis in rabbit pups. However, there was no evidence of increased cell death in glucocorticoid-exposed human infants.

Conclusions—Prenatal glucocorticoid suppresses vascular endothelial growth factor and elevates transforming growth factor-β levels, which results in angiogenic inhibition, trimming of neovasculature, and enhanced pericyte coverage. These changes contribute to stabilizing the germinal matrix vasculature, thereby reducing its propensity to hemorrhage. Prenatal glucocorticoid exposure does not induce neural cell death in humans, unlike rabbits. (Stroke. 2010;41:1766-1773.)

Key Words: betamethasone □ germinal matrix hemorrhage–intraventricular hemorrhage □ germinal matrix glucocorticoids □ pericyte □ TGF-β □ vasculature

Prenatal glucocorticoids (GCs) prevent respiratory distress syndrome and intraventricular hemorrhage (IVH) in preterm infants.1,2 Indeed, the National Institutes of Health Consensus Development Panel on the “Effect of corticosteroids for fetal maturation on perinatal outcomes” has recommended use of prenatal GC in preterm labor.3 In the United States, the preterm birth rate is 12.5%, and 75% of women in preterm labor with gestational age of ≤34 weeks are treated with GC.4 Because approximately 13 million babies are born premature worldwide every year, a huge number of preterm infants are exposed to prenatal steroids. This increases their survival and reduces both the incidence and severity of IVH.5,6 Yet, the molecular mechanism by which GCs prevent IVH is elusive. Therefore, we asked how prenatal GC would reduce the incidence of IVH.

IVH typically initiates in the germinal matrix (GM). This periventricular region, located on the head of the caudate nucleus and underneath the ventricular ependyma, is a richly vascularized collection of neural precursor cells and is selectively vulnerable to hemorrhage. IVH is attributed to intrinsic fragility of the GM vasculature and disturbance in cerebral blood flow. Our previous work has shown that a rapid angiogenesis in the GM, induced by high vascular endothelial growth factor (VEGF) and angiopoietin (ANGPT)-2 levels, contributes to increased vascular fragility and vulnerability to hemorrhage and that angiogenic inhibition reduces the occur-
rence of IVH in a rabbit pup model. Furthermore, angiogenic vessels of the GM exhibit paucity of pericytes, deficiency of fibronectin in the basal lamina, and reduced perivascular coverage by glial fibrillary acidic protein (GFAP)-positive astrocyte end feet. Hence, the fragility of GM microvasculature is attributed to immature basal lamina and reduced perivascular coverage by pericytes as well as GFAP-positive end feet.

Prenatal GC betamethasone and its stereoisomer, dexamethasone, are used in preterm labor. They exhibit a wide range of pharmacological effects and toxicities on the brain of premature infants. However, little is known about the effects of GCs on the morphology and molecular components of the developing cerebral vasculature. The GC downregulates VEGF in an in vitro model of the blood–brain barrier and cultured cells of various origin and, accordingly, the GC treatment effectively suppresses angiogenesis in various disease models. The blockade of VEGF signaling prunes the nascent, immature, and pericyte-deficient microvasculature of tumors. In addition, this remodels the remaining vasculature, which results in less dilated blood vessels exhibiting enhanced pericyte coverage. Other than VEGF, growth factors angiopoietin-1, platelet-derived growth factor-B (PDGF-B), and transforming growth factor-β (TGF-β) play a key role in maturation of the vasculature, particularly in the assembly of pericytes around the immature blood vessels. Therefore, we hypothesized that prenatal GC would suppress angiogenesis by downregulation of angiogenic growth factors, including VEGF and ANGPT-2, and enhance pericyte recruitment by inducing distinctive changes in the regulating growth factors angiopoietin-1, PDGF-B, and TGF-β.

There is increasing evidence that the GC treatment affects the phenotype and function of astrocytes. For example, the dexamethasone treatment in the astrocyte cultures and triamcinolone intravitreal injection in a mice model of laser retinal damage augments the phenotype and function of astrocytes. For example, the fragility of GM microvasculature is attributed to immature basal lamina and reduced perivascular coverage by pericytes as well as GFAP-positive end feet. After stabilization of their conditions, they were weighed and fed with puppy formula (Esbilac; Petag, Hampshire, Ill). Pups were euthanized at 3 epochs: 2, 6, and 48 hour of age. Brain was then dissected and cut into 2-mm coronal slices on brain matrix. All the histological evaluations were done from coronal sections taken at the level of midseptal nucleus. The comparison groups were balanced with respect to the body weight and gender of rabbit pups.

**Laser Capture Microdissection**


**Human Tissue Collection and Processing**

The Institutional Review Board of New York Medical College approved the use of human autopsy materials for this study. Women in preterm labor receive either betamethasone (12.5 mg once daily for 2 days) or dexamethasone (6 mg twice daily for 2 days) to prevent respiratory distress syndrome in premature infants. The preterm infants included in the present study delivered within a few hours to 3 days after completion of GC treatment to their mothers; and infants died at 6 to 72 hours of postnatal age (Supplemental Table I, available online at http://stroke.ahajournals.org). The wall of the cerebral hemisphere in fetuses consists of ventricular zone, subventricular zone, intermediate zone, cortical plate, and marginal zone as described by the Boulder Committee. In this study, we described intermediate-zone embryonic white matter synonymously with white matter and cortex for the cortical plate for the sake of simplicity of presentation. Brain samples were processed as described. Approximately 2- to 3-mm thick coronal slices were taken at the level of thalamostriate groove from the frontal lobe. The coronal blocks included the frontal lobe cortex, white matter, and GM. The samples were fixed in 4% paraformaldehyde in phosphate-buffered saline for 18 hours and then were cryoprotected by immersing into 20% sucrose in phosphate-buffered saline buffer. The tissues were frozen after embedding them into optimum cutting temperature compound. Frozen coronal blocks were cut into 15-μm sections using a cryostat and saved at −80°C until use.

**Immunohistochemistry, Neuronal Degeneration**

(Fluoro-Jade B), Fluorescent In Situ Detection of DNA Fragmentation (Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick End Labeling), Western Blot Analyses, and Quantitative Real-Time Polymerase Chain Reaction

The techniques are illustrated in the Supplemental Methods.

**Quantification of Vascular Density, Endothelial Proliferation, Cell Death, Pericyte, and Astrocyte Coverage**

These are described in the Supplemental Methods.

**Statistical Analysis**

To determine differences in the endothelial proliferation, vascular density, vessel area, and cell degeneration between GC-exposed and unexposed human infants, 2-way analysis of variance with repeated measures was used. The repeated factor was applied to the 3 brain regions: cortex, white matter, and GM. To assess differences in endothelial proliferation, vessel density, vessel area, pericyte coverage, and growth factors in rabbit pups, 2-way analysis of variance was used for each of the brain regions (cortex, white matter, and GM) separately. The independent factors in 2-way analysis of variance were postnatal age (2 hours versus 48 hours) and treatment (betamethasone versus no treatment). All post hoc comparisons to test for differences between means were done using Tukey multiple comparison test at the 0.05 significance level. Student t test was used to compare between 2 groups (Western blot analyses data).
Results

Glucocorticoids Reduce Endothelial Proliferation in Both Rabbits and Humans

Because GC suppresses angiogenesis in various disease models,16,17 we asked whether GC would reduce endothelial proliferation in the GM. To this end, we double-labeled the brain sections with Ki67 (proliferation marker) and CD31 (endothelium in rabbit) or CD34 (endothelium in human)–specific antibodies and evaluated x-z and y-z (orthogonal views) reconstructions of stacks of confocal images to verify Ki67 immunoreactivity embedded into CD34 (Figure 1A, C).

The endothelial proliferation index was significantly lower in the GM of betamethasone-treated rabbit pups compared with untreated controls at both 2 and 48 hours of age (***P<0.001, **P<0.01); and this decreased at 48 hours than at 2 hours of age in both control and treated pups (###P<0.001, †P<0.05). Cryosections from 23-week premature infants exposed and unexposed to GC labeled for Ki67 and CD34 antibodies. Note endothelial proliferation (Ki67 overlapping CD31, arrowhead) more frequent in GC-treated infants than in controls. Scale bar, 50 μm. D, Data are mean±SEM (n=5 at each point). Endothelial proliferation was less in GM of GC-treated infants relative to untreated controls (*P<0.05).

Glucocorticoids Prune the GM Vasculature in Both Rabbits and Humans

Because GC suppresses VEGF expression in the culture experiments14 and because VEGF inhibitors destroy the angiogenic vasculature in tumors,18 we compared vascular density and percent blood vessel area between coronal sections (midseptal nucleus level) of betamethasone-treated pups and untreated controls. We found that the vascular density was significantly reduced in the GM of betamethasone-treated rabbit pups compared with untreated controls at both 2 and 48 hours of age (P<0.001 and 0.007, n=5 each group at each epoch; Figure 2A). The endothelial proliferation index was also less abundant at 48-hour age than at 2-hour age in both control and betamethasone-treated pups (P<0.001 and 0.017, respectively). The endothelial turnover in the cortex and white matter was significantly fewer relative to the GM at 2 hours of age (data not shown).

We next compared endothelial proliferation between human premature infants exposed and unexposed to prenatal GC, betamethasone or dexamethasone (Supplemental Table I). The infants in the 2 groups were of comparable gestational (23 to 25 weeks) and postnatal age (<72 hours). Similar to rabbit pups, prenatal GC exposure significantly reduced the endothelial proliferation index in the GM (P=0.019, n=5 each, Figure 1D) of premature infants. In the cortex and white matter, the endothelial proliferation was significantly less compared with the GM in both GC-treated and untreated groups (data not shown) and did not reduce on exposure to prenatal GC. Together, prenatal GC exposure diminished endothelial proliferation in the GM of both the premature rabbit pups and human infants.

Figure 1. GC suppresses endothelial proliferation. A, Representative immunofluorescence of cryosections from E29 rabbit pups stained with Ki67 and CD31 antibodies. Note CD31 staining vessels with Ki67 signals indicating endothelial proliferation (arrowhead). Vessels with Ki67 labeling were fewer in the betamethasone-treated pups than controls. Above and right to the image are orthogonal views in x-z and y-z planes of a composite of z-stack of a series of confocal images taken 0.6 μm apart. Scale bar, 20 μm. B, Data are mean±SEM (n=5 at each point). Endothelial proliferation was lower in betamethasone-treated pups than in controls at both 2 and 48 hours of age (***P<0.001, **P<0.01); and this decreased at 48 hours than at 2 hours of age in both control and treated pups (###P<0.001, †P<0.05). C, Cryosections from 23-week premature infants exposed and unexposed to GC labeled for Ki67 and CD34 antibodies. Note endothelial proliferation (Ki67 overlapping CD31, arrowhead) more frequent in GC-treated infants than in controls. Scale bar, 50 μm. D, Data are mean±SEM (n=5 at each point). Endothelial proliferation was less in GM of GC-treated infants relative to untreated controls (*P<0.05).
significantly less in the GC-treated infants than in untreated controls ($P<0.04$ and $0.008$, respectively; Figure 2C–D). In the cortex and white matter, these metrics were comparable between the treatment and control groups. Hence, GC trims the angiogenic GM vasculature in the premature rabbit pups and human infants.

**Betamethasone Suppresses VEGF But Not ANGPT-2**

Because prenatal GC pruned GM neovasculature in our experiments, we asked whether prenatal betamethasone would suppress the angiogenic growth factors, VEGF and ANGPT-2, in the GM. To this end, we measured protein levels of these 2 growth factors in homogenates from a coronal slice taken at the midseptal nucleus level; and we assayed mRNA expression in the laser dissected samples from the 3 brain regions: GM, cerebral cortex, and white matter. Western blot analysis revealed that 24 kDa VEGF was significantly less abundant in betamethasone-treated rabbit pups compared with untreated controls ($P=0.04$; Figure 3A). Accordingly, real-time polymerase chain reaction showed that betamethasone treatment reduced VEGF mRNA level in the GM ($P=0.02$; Figure 3B) but not in the cortex and white matter at 2 hours of age. At 6 hours of age, VEGF mRNA expression was significantly reduced in all the 3 brain regions—cortex, white matter, and GM—of the betamethasone-treated pups compared with controls ($P=0.01$, $0.04$ and $0.02$, respectively). However, prenatal

**Figure 2.** GC reduces vascularity of the germinal matrix. A–B, Data are mean±SEM (n=5 at each point). Vascular density was less in the GM of GC-exposed pups compared with unexposed controls at 2 hours but not at 48 hours of age ($P<0.05$ each). The percent blood vessel area in the GM was smaller in betamethasone-treated pups at 2 hours of age than in untreated controls ($P<0.01$), but not at 48 hours of age. The percent blood vessel area is also lesser at 48 hours of age than at 2 hours of age in control pups (##$P<0.01$). Data are mean±SEM (n=5 at each point). Both the vascular density and percent vessel area in the GM were less in GC-treated infants than in untreated control infants ($P<0.05$, **$P<0.01$). Beta indicates betamethasone.

**Figure 3.** Prenatal betamethasone down-regulates VEGF but not ANGPT-2. A, Representative Western blot analyses of VEGF from a coronal slice of the forebrain. VEGF levels normalized for $\beta$ actin. Data are mean±SEM (n=5 at each point). VEGF was significantly less abundant in betamethasone-treated rabbit pups compared with untreated controls ($P<0.05$). B, Data are mean±SEM (n=5 at each point). Beta-methasone treatment reduced VEGF mRNA level in the GM at both 2 and 6 hours of age ($P<0.05$ each). This also reduced the VEGF level in the cortex and white matter at 6 hours of age (**$P<0.01$, *$P<0.05$). C, Representative Western blot analyses of ANGPT-2 from a coronal slice of the forebrain. ECV 304 cell lysate (Santa Cruz, Calif) was used as positive control. Data are mean±SEM (n=5 at each point). ANGPT-2 are similar between betamethasone-treated and control pups. D, Data are mean±SEM (n=5 at each point). Betamethasone did not affect ANGPT-2 mRNA levels.
Betamethasone treatment did not affect VEGF mRNA levels at 48 hours of age in any of the brain regions. Importantly, ANGPT-2 protein and mRNA accumulation were similar in the treated and control pups (Figure 3C–D). Together, betamethasone treatment suppressed VEGF but not ANGPT-2.

**Betamethasone Enhances Pericyte Coverage in the GM Vasculature**

There is paucity of pericytes in the GM vasculature; and VEGF inhibition prunes the immature vessels lacking pericytes. Therefore, we postulated that betamethasone treatment would enhance pericyte coverage in the GM vasculature. Thus, we assessed coronal brain sections double-labeled with NG2 (pericyte marker) and CD31 antibodies. In rabbits, we found that betamethasone enhanced pericyte vascular coverage at both 2 and 48 hours of age ($P<0.001$ each; Figure 4A–B) in the GM, but not in the cortex or white matter. However, the pericyte coverage remained significantly less in the GM than in the other brain regions in the treated pups.

We next evaluated pericyte coverage in human autopsy materials from premature infants and obtained similar findings as in rabbit pups. The pericyte coverage was higher in the GM of premature infants exposed to prenatal GC compared with untreated infants ($P=0.016$; Figure 4C–D). GC exposure did not affect pericyte coverage in the cortex and white matter. However, the pericyte coverage remained significantly less in the GM than in the other brain regions in the treated pups.

**Betamethasone Elevates TGF-$\beta$ But Not ANGPT-1 or PDGF-B Levels**

The ligand receptor systems that recruit pericytes include TGF-$\beta$, ANGPT-1, PDGF-B, and their receptors. Because betamethasone treatment augmented pericyte coverage in the GM vasculature, we determined whether TGF-$\beta$, ANGPT-1, and PDGF-B levels were higher in the GM of GC-treated pups compared with untreated controls. Real-time polymerase chain reaction showed that ANGPT-1 and PDGF-B levels were comparable between betamethasone-treated and control groups at all epochs (Figure 5A–B). However, TGF-$\beta$ mRNA expression was elevated in the rabbit GM exposed to betamethasone compared with unexposed controls at both 2 and 6 hours ($P=0.04$ and 0.03, respectively), but not at 48 hours of age. TGF-$\beta$ was also higher in the cortex of treated pups compared with controls at 2 hours ($P<0.05$) but not at 6 and 48 hours of age (Figure 5C). To confirm elevation in TGF-$\beta$ levels, we measured its protein expression by Western blot analyses and found that TGF-$\beta$ protein level was higher in betamethasone-treated rabbit pups compared with untreated controls ($P<0.05$; Figure 5D). Hence, betamethasone treatment upregulates TGF-$\beta$ levels but not ANGPT-1 or PDGF-B expression.

We also assessed the receptors of VEGF, ANGPT, and PDGF-B by real-time polymerase chain reaction. We found no significant difference in mRNA expression of VEGF-R2, Tie-2, and PDGF-Ra receptors between betamethasone-treated and control groups (data not shown).

**Betamethasone Enhances GFAP-Positive Astrocytes in the GM**

Because GC treatment enhances GFAP in astrocytes, we assessed GFAP expression in the GM using immunohistochemistry. Immunolabeling revealed that GFAP-positive astrocytes were more abundant in the GM of betamethasone-treated pups compared with untreated controls at both 2 and 48 hours of age (Figure 6A). We next performed Western blot analyses on the homogenates from 1-mm thick brain slice at the level of midseptal nucleus. GFAP protein expression was significantly greater in the betamethasone-treated pups compared with untreated controls ($P<0.05$, $n=5$ in each group; Figure 6B).

We then compared GFAP-positive perivascular end feet coverage in the GC-exposed and unexposed human infants. The percent GFAP-positive astrocyte end feet was 1.5-fold greater in the betamethasone-treated pups compared with
untreated controls (32.5±9.6% versus 20.2±4.3%). The difference, however, was not statistically significant. In conclusion, prenatal GC significantly enhances GFAP expression in the astrocytes of the rabbit GM but not GFAP-positive end feet coverage in the GM of premature infants.

**Betamethasone Induces Apoptosis, Neuronal Degeneration, and Growth Retardation in Rabbits**

Because betamethasone treatment can induce cell death, we assessed apoptosis and neuronal degeneration in rabbit pups treated with prenatal betamethasone compared with untreated controls at 2 hours postnatal age. Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL)-positive cells were more abundant in the GM, cortex, and white matter of the treated pups compared with untreated controls (P<0.001, 0.025, and 0.026, respectively; Figure 6 and Supplemental Figure I, available online at http://stroke.ahajournals.org). Accordingly, Fluoro-Jade B-positive neurons were in larger number in the GM, cortex, and white matter of treated pups compared with untreated controls (P=0.001, 0.022, and 0.003 respectively; Figure 6D, Supplemental Figure I). We next assessed apoptosis and neuronal degeneration in human premature infants exposed and unexposed to antenatal GC. In contrast to rabbits, density of TUNEL-positive neural cells and Fluoro-Jade B-positive neurons were remarkably comparable between the 2 groups of human infants (Supplemental Figure I).

The betamethasone-treated pups were markedly smaller in weight (29.3±1.6 g versus 47.1±0.9 g; P<0.001, n=12 pups each) compared with untreated controls. Hence, prenatal GC...
induces cell death in premature rabbit pups but not in preterm infants.

**Discussion**

IVH is the most common neurological complication of prematurity affecting approximately 12,000 infants each year in the United States alone. Of note, prematurity rate is escalating; survival of premature infants has remarkably increased with advances in the medical care; and the IVH rate among preterm infants has remained almost stationary during the last 10 years. Thus, IVH and the attendant complications, including cerebral palsy, posthemorrhagic hydrocephalus, and cognitive deficits, have emerged as global health problems. No treatment of IVH is currently available. The only widely practiced preventive strategy is the use of prenatal GC in women in preterm labor, which reduces the occurrence of IVH in preterm infants by >50%. In this study, we determined the mechanistic basis of the use of prenatal GC to prevent IVH in preterm infants. We found that the prenatal GC suppressed angiogenesis, pruned the neovasculature, and enhanced pericyte coverage, thereby stabilizing the GM vasculature. We then observed that prenatal GC increased apoptotic neural cell death and neuronal degeneration in premature rabbit pups but not in human premature infants.

In the present study, the GC suppressed VEGF expression in the forebrain, particularly in the GM; and accordingly, endothelial proliferation was diminished in the GM of GC-treated pups compared with untreated controls. Consistent with our findings, the GC downregulates VEGF in a culture model of the blood—brain barrier as well as in other cell types; and GC also suppresses tumor angiogenesis in animal models. Importantly, our previous study has shown that GM has high VEGF and angiopoietin-2 levels inducing rapid endothelial proliferation in the microvasculature and that the suppression of VEGF by celecoxib, a cyclo-oxygenase-2 inhibitor, or ZD6474, a VEGFR2 blocker, minimizes both the incidence and severity of IVH. Therefore, this is plausible that the prenatal GC confers protection against IVH by downregulating VEGF and suppression of angiogenesis.

Another key finding in our study was that the GC treatment reduced vascular density and enhanced pericyte coverage in the GM microvasculature. Because angiogenic inhibitors suppress VEGF levels resulting in apoptosis of endothelial cells not protected by pericytes, the increase in pericyte coverage on GC exposure could be secondary to selective loss of neovascularization lacking in pericytes. Importantly, trimming of GM vasculature might impair oxygenation in this brain region that could adversely affect its development. To determine an alternate mechanism that might augment pericyte coverage in the vasculature, we assessed levels of growth factors involved in pericyte recruitment. We observed elevation in TGF-β levels on GC treatment, but not in PDGF-B, angiopoietin, or their receptors: Tie-2 and PDGFRβ. TGF-β promotes stabilization of the neovasculature by differentiation of pericytes from mesenchymal cells and by recruitment of pericytes around the angiogenic blood vessels. TGF-β in the GM with GC exposure might assist in suppression of angiogenesis and contribute to pericyte recruitment in the GM vasculature.

Our previous work has shown that perivascular coverage by GFAP-positive end feet was decreased in the GM compared with the cerebral cortex and white matter in premature infants 23 to 34 weeks. We expected that GC exposure will increase GFAP-positive end feet in the GM. However, although GFAP-positive end feet perivascular coverage tended to be elevated in the GM of infants exposed to GC, the difference was not statistically significant. These studies performed on human autopsy material of a short postmortem interval are invaluable. Nevertheless, the limitations of such studies are exposure of infants to a number of prenatal and postnatal variables, including mechanical ventilation, mediations, and others, which can potentially confound the results. We also found elevation in GFAP levels in the rabbit brain, demonstrated by immunolabeling and Western blot analyses. The increase in GFAP on GC treatment might be attributable to an elevation in TGF-β levels. Several other studies have also shown an elevation in GFAP levels in astrocytes on steroid treatment. Together, GC treatment enhances GFAP levels in the astrocytes, but this elevation may not be significant in the perivascular end feet.

Of note, we observed abundance of apoptotic neural cells and neuronal degeneration in the rabbit pups exposed to prenatal GC. High doses of dexamethasone and methylprednisolone also induce apoptotic cell death in hippocampal culture experiments and in rats and monkeys. The apoptosis is typically mediated by GC receptors through genomic or nongenomic pathways, and these effects differ with respect to GC preparation, dose and duration of treatment as well as with the stage of neural cell maturation. In contrast to rabbit pups, cell death was comparable in premature infants exposed and unexposed to prenatal GC. Similar to humans, prenatal dexamethasone exposure does not affect neural cell death in ovine fetuses at 90% gestation. However, at 70% gestational age, prenatal dexamethasone reduces apoptosis and caspase activity in the ovine fetal cerebral cortex. This marked discrepancy in the effect of GC on cell death among the human, rabbit, and sheep fetuses could be attributed to distinctive maturation and susceptibility of neural cells to GC as well as to the differences in the pharmacokinetics of GC among the species. All the mothers of the infants included in the present study completed the GC course within 72 hours of the delivery of their infants; and these premature infants died at 6 to 72 hours postnatal age. Therefore, it is less likely that we missed the window of apoptotic cell death and neuronal degeneration after prenatal steroid treatment. In this context, it is important to link intrauterine cerebral development of rabbits to humans. The E29 rabbit pups (term = 32 days, E29 = 85% to 90% gestation) could be considered equivalent to 33-week premature infant. However, previous studies indicate that cortical and noncortical development of E29 rabbits equates to approximately 20 weeks of gestational age in humans and myelination initiates in the early third trimester in humans and at postnatal Day 4 to 7 in rabbits. Thus, E29 pups might be similar to premature infants of 30±4 weeks gestational age.
This article presents the mechanistic basis of GC treatment in the prevention of IVH. Obtaining autopsy materials from premature infants of a short postmortem interval with comparable demographics for the GC treatment and a control group was a result of our diligently made, unremitting effort of several years. The infants in the 2 groups were of short postnatal age to reflect the effect of prenatal steroid. However, the limitations of human studies are exposure of premature infants to a number of pre- and postnatal variables, including mechanical ventilation, exposure to medications, and others, that can potentially impact the data. The data in both rabbits and humans showed that GC augmented the perivascular pericyte coverage. However, despite the enhancement in pericyte coverage after GC treatment, this remained less in the GM than in the other brain regions: cerebral cortex and white matter. Because pericytes are the providers of structural integrity to the vasculature, strategies to further enhance the pericyte coverage might offer greater protection against the development of IVH in premature infants.

In conclusion, prenatal GC suppressed VEGF levels and elevated TGF-β, which resulted in inhibition of angiogenesis, trimming of the neovasculature, and enhancement in the pericyte coverage. These morphological and molecular changes would stabilize the GM vasculature, thereby reducing its vulnerability to hemorrhage. Prenatal glucocorticoid exposure did not induce neural cell death in premature human infants, unlike rabbit pups.

Acknowledgments
We thank Qihui Shi, PhD, for statistical advice.

Sources of Funding
Supported by an American Heart Association grant-in-aid and National Institutes of Health/National Institute of Child Health and Human Development HD061778 (to P.B.).

Disclosures
None.

References


Supplemental Methods

Laser Capture Microdissection

Because GM of rabbit pup brain cannot be dissected accurately with the naked eye because of its microscopic size, we used laser capture microdissection to collect samples from rabbit GM, cortex, and white matter. Frozen sections of 10 μm thickness were cut using cryostat and sections were mounted on RNase-free membrane slides (PALM microlaser technologies; Zeiss Inc, Thornwood, NY) and stored at −80°C. Frozen sections, obtained on membrane slides, were stained by hematoxylin. Microdissection of the GM, cortex, and white matter was performed using the PALM microlaser technology as described.7

Immunohistochemistry

The primary antibodies used in experiments included mouse monoclonal CD34 QBEnd/10 (catalog 326-01; Covance, Emeryville, Calif), goat polyclonal CD31/PECAM-1 (catalog sc-1505; Santa Cruz Biotech, Santa Cruz, Calif), NG2 mouse monoclonal (catalog mab2029; Millipore, Temecula, Calif), mouse monoclonal GFAP (catalog # G3893; Sigma, St Louis, Mo), mouse monoclonal VEGF (catalog sc 152; Santa Cruz Biotech), goat polyclonal ANGPT-2 (catalog sc-7017; Santa Cruz Biotech), mouse monoclonal Ki67 (catalog 550609; BD Biosciences, San Jose, Calif), and rabbit monoclonal Ki67 (catalog 275R-14; Cell Marque, Rocklin, Calif). Secondary antibodies, including fluorescein isothiocyanate conjugate goat–antimouse, rhodamine conjugate goat antirabbit, and cy-5 conjugate goat–antirabbit, were bought from Jackson Immuno-research (West Grove, Pa). Briefly, the sections were incubated with 5% NGS, 1% beef serum albumin, and 0.2% Triton X-100 in phosphate-buffered saline for 2 hours at room temperature. The tissue sections then were incubated overnight at 4°C with primary antibodies diluted in phosphate-buffered saline. After several washes in phosphate-buffered saline, sections were incubated in secondary antibodies for 2 hours at room temperature. Finally, after washes in phosphate-buffered saline, sections were mounted with Slow Fade Light Antifade reagent (Molecular Probes, Calif).

Endothelial Proliferation Index

For the assessment of proliferating endothelial cells, rabbit brain sections were double-labeled with mouse monoclonal Ki67 nuclear antigen (BD Biosciences) and platelet–endothelial cell adhesion molecule-1 (CD31)-specific antibodies. In humans, we used Ki67 (Cell Marque) and CD34 (Covance)-specific antibodies to evaluate endothelial turnover. Ki67 nuclear antigen detects cells in late G1, S, G2, and M phases of the cell cycle. Endothelial proliferation index was calculated as percentage of the ratio of all Ki67-positive immunoreactivity embedded within CD34 (CD31 staining in rabbits) positive blood vessels and total number of CD34 (CD31 in rabbits)-positive vessels.

Neuronal Degeneration and Fluorescent In Situ Detection of DNA Fragmentation (TUNEL)

We performed Fluoro-Jade B (Chemicon) and TUNEL staining on fixed brain sections as described previously.32 For TUNEL staining, 15-μm tissue sections were air dried on slides, hydrated in 0.01 mol/L phosphate-buffered saline, and permeabilized for 5 minutes in 1:1 ethanol-acetic acid. An ApopTag-fluorescein in situ DNA fragmentation detection kit (Chemicon) was used to visualize TUNEL-labeled nuclei.

Figure 1. Prenatal GC induces cell death in rabbit pups but not in human infants. Representative TUNEL and Fluoro-Jade B staining of brain sections from premature rabbit pups (E29) and premature infants (23 to 24 weeks gestation) exposed (upper panel) and unexposed (lower panel) to prenatal GC. TUNEL-positive and Fluoro-Jade B-positive cells (arrowhead) were abundant in rabbit pups treated with prenatal GC, whereas these cells were scarce in pups unexposed to GC. In human infants, TUNEL-positive cells were scant and Fluoro-Jade B-positive cells were rare both in premature infants exposed and unexposed to prenatal GC. Scale bar, 50 μm. Insets show high-power view of TUNEL-positive and Fluoro-Jade B-positive cells in rabbit pups exposed to GC. Scale bar, 20 μm.
Quantification of Vascular Density, Endothelial Proliferation, Cell Death Pericyte, and Astrocyte Coverage

We counted number of vessels, endothelial proliferation, TUNEL-positive nuclei, and degenerated neurons in coronal brain sections of GC-exposed and untreated rabbit pups and human infants. From each brain, 5 coronal sections (30 μm) taken as every third section at the level of midseptal nucleus were used for the study. Counting was performed in an unbiased fashion and random basis in the GM, cortex, and white matter by 2 blinded investigators using a BIO-RAD (MRC-1024ES) confocal microscope. We counted objects in approximately 150 images (7 to 10 images/3 brain regions/5 coronal sections) per brain (n=5 subjects each group) for each parameter.

Pericyte and Astrocyte End Feet Coverage

Images were analyzed using Metamorph Version 6.1 from Universal Imaging Corporation 1993 to 2003 (Downington, Pa) as previously described.8,9 Briefly, 2-dimensional images were acquired by confocal microscope using 60x objective and were analyzed for the overlapping of the perimeter of blood vessels on pericyte or astrocyte end feet. The 2 sources of image, endothelium (red) and pericyte/astrocyte end feet (green), were displayed on the Metamorph screen. Both images were thresholded. “Region tool” in the software was next used to define and select the outer margin of the blood vessel. The software measured this delineated area in pixels and calculated the percentage overlap of red (endothelium) over green signal (pericyte or astrocytes end feet). We analyzed blood vessel profile in 4 to 5 coronal sections taken as the third section at the level of midseptal nucleus in rabbit pups and head of the caudate nucleus in human infants (n=5 each).

Western Blot Analyses

Protein extraction and Western blot for VEGF, ANGPT-2, and TGF-β were performed under reducing conditions as described before.7 Briefly, from the forebrain of E29 pups, we took 2-mm thick slices at the midseptal nucleus level. The protein concentration in the sample was determined using a RC DC protein assay kit (Biorad). Equal amounts of protein (20 μg) were loaded into 4% to 15% gradient precast gel (Bio-Rad). The separated proteins were transferred to polyvinylidene difluoride membrane by electrotransfer. The membranes were then incubated with primary antibodies, washed, incubated with secondary antibody, and washed again. Target proteins were detected with the chemiluminescence ECL system (Amersham).

Quantitative Real-Time Polymerase Chain Reaction

The total RNA from the rabbit cortex, white matter, and GM, which was dissected by laser capture microdissection, was isolated using Mini RNA isolation kit (Zymo Research) according to the manufacturer’s protocol. The total RNAs from rabbit were reverse-transcribed to cDNAs by using SuperScript First strand Synthesis System for real-time polymerase chain reaction kit (Invitrogen) following the manufacturer’s protocol. Real-time polymerase chain reaction was used to analyze mRNA expression using the Stratagene MX3000 (Bertried, Germany) on the cDNA as described.7 Samples were run in triplicates. Quantification was performed using the ∆∆Ct method. The housekeeping gene, GAPDH, was used for internal normalization.
Effect of Prenatal Glucocorticoids on Cerebral Vasculature of the Developing Brain
Govindaiah Vinukonda, Krishna Dummula, Sabrina Malik, Furong Hu, Carl I. Thompson, Anna Csiszar, Zoltan Ungvari and Praveen Ballabh

Stroke. published online July 8, 2010;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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