Intraventricular hemorrhage, or hemorrhage into the germinal matrix tissues of the developing brain, remains a common problem of preterm infants. The "risk period" for this insult is the first 3–4 postnatal days. We hypothesized that this risk period for hemorrhage is related to rapid perinatal maturation of the germinal matrix vasculature and employed the newborn beagle pup model for the study of this maturation. Newborn beagle pups (n=30) were anesthetized and systemically perfused with buffered formalin; the brains were removed and prepared for immunohistochemical study. Sections stained with Bandeiraea lectin demonstrated that there was no difference in germinal matrix vessel density between postnatal days 1 and 4. Germinal matrix sections were also stained for antibodies to α-smooth muscle actin, collagen IV, collagen V, desmin, factor VIII-related antigen, fibronectin, glial fibrillary acidic protein, laminin, transferrin, and vimentin. Vasculature staining by α-smooth muscle actin was not noted until postnatal day 10, and differential staining was detected for antibodies to laminin and collagen V. Quantification of staining intensity by confocal microscopy demonstrated a significant increase in both extracellular matrix components at postnatal day 4 compared with day 1 (p<0.05 for both). These basement membrane proteins may add sufficient structural integrity to germinal matrix vessels to prevent capillary rupture and thus intraventricular hemorrhage.

Pregnant beagles were obtained 2 weeks prior to their expected whelping date. The date and time of birth of each litter of pups were noted, and pups from four litters were employed in the following studies. Pups from each litter were sacrificed on PNDs 1, 4, or 10.

The pups (n=30) were anesthetized with 30 mg/kg pentobarbital. After they were unresponsive to deep noxious stimulation, a thoracotomy was performed and the pups were systemically perfused with buffered formalin. The brains were removed and placed in buffered formalin overnight. The following day the brains were placed in 0.1 M phosphate buffer with 20% sucrose and allowed to remain in this solution for up to 1 week.

The brains were serially sectioned at 80 μm using a freezing microtome and were then placed in a cryoprotectant solution and stored at −20°C until used for lectin or antibody staining.

Multiple tissue sections from three or four animals from different litters at PND 1, 4, and 10 were stained with Bandeiraea, Lycopersicon, and Ulex lectins from Sigma Chemical Co., St. Louis, Mo. Tissue sections were incubated overnight in the lectins at a concentration of 0.01 mg/ml in phosphate-buffered saline (pH 7.2) with 3% bovine serum albumin and 0.01% Triton X-100 (Sigma). The following day the sections were washed with phosphate-buffered saline (pH 7.2) without azide and reacted with freshly prepared 0.03% diaminobenzidine tetrahydrochloride with 0.03% H2O2. The sections were then washed with phosphate buffered saline (pH 7.2) and mounted on glass slides, dehydrated, and coverslipped.

The Bandeiraea lectin–stained sections were used for determining vessel density of the cortical, periventricular white matter, and germinal matrix regions. For this protocol, photographic slides made from low-power photomicrographs were projected on a standardized grid and the vessel density was measured.

Tissue sections from three or more animals from different litters at each PND were also stained with antibodies to the proteins α-smooth muscle actin, α2-macroglobulin, desmin, glial fibrillary acidic protein, fibronectin, transferrin, vimentin (all from Sigma), endothelin (Peninsula Laboratories, Inc., Belmont, Calif.), prostaglandin F1α (ICN Biomedicals, Inc., Lisle, Ill.), factor VIII–related antigen (Dakopatts, Glostrup, Denmark), laminin, collagen IV, and collagen V (all supplied by Dr. J.A. Madri).18 Tissue sections were incubated in antibodies prepared at concentrations ranging from 1:20 through 1:500 in 3% bovine serum albumin/phosphate-buffered saline with 0.2% azide and 0.001% Triton X-100. The following day, the tissue sections were washed with phosphate-buffered saline (pH 7.2) and then incubated for 3 hours in horseradish peroxidase– or tetramethylrhodamine isothiocyanate–labeled secondary antibodies (1:100 concentration, Sigma). Following this incubation, the sections were washed with phosphate-buffered saline (pH 7.2) three times, and the horseradish peroxidase–labeled sections were reacted with freshly prepared diaminobenzidine tetrahydrochloride and prepared as above. The sections stained with fluorescent-labeled secondary antibodies were washed three times with phosphate-buffered saline (pH 7.2) and mounted on glass slides, dehydrated, and coverslipped.

Confocal microscopy was performed using an MRC-500 scanning laser microscope (Bio-Rad, Cambridge, Mass.) To achieve consistency in sampling technique, the variable confocal aperture was set to a predetermined setting to give an optical section thickness exhibiting an acceptable signal-to-noise ratio. All subsequent samples were viewed at this setting using the same objective lens (×16) and raster-controlled zoom factor (×2). Images thus created possess identical “volumes” of fluorescence signal and can then be analyzed using the MRC system software for pixel intensity and area to quantify relative changes between tissues from animals of different postnatal ages.

Confocal microscopy was performed on multiple sections of germinal matrix and periventricular white matter regions from four animals from different litters at each postnatal age.

Student's unpaired t test was used for analysis of intensity and density data.

Results

The lectins from Bandeiraea and Lycopersicon stained microvessels in the cortical, periventricular white matter, and germinal matrix regions from pups of all three postnatal ages examined equally well. No staining with Ulex lectin was noted in the cerebral microvessels of any region at any postnatal age. Based on these data, Bandeiraea lectin was chosen as the stain for determining vessel density (Figure 1).

Vessel density was determined for the germinal matrix, periventricular white matter, and cortical regions of at least three pups from different litters at each postnatal age. The data for these measurements is found in Table 1 and demonstrate that although there is no difference in vessel density for the germinal matrix, periventricular white matter, or cortex between PND 1 and PND 4, there is a significant decrease in germinal matrix vessel density at PND 10 compared with either PND 1 or PND 4 (p<0.05 for either comparison). There is also a significant decrease in cortical vessel density at PND 10 (p<0.05 different from either PND 1 or PND 4) but no difference in periventricular white matter vessel density.

Germinal matrix vessel antibody staining data are summarized in Table 2. Differential microvasculature staining for the three postnatal ages examined was detected only for antibodies to α-smooth muscle actin, collagen V, and laminin. Monoclonal antibodies to these epitopes were present at PND 1 and increased markedly at PND 4. Germinal matrix microvessels stained with antibodies to laminin are shown in Figure 2. In contrast, monoclonal antibody staining to α-smooth muscle actin was not noted in the germinal matrix until PND 10.
Germinal matrix microvessel staining for antibodies to α2-macroglobulin, endothelin, factor VIII-related antigen, glial fibrillary acidic protein, and vimentin was found in pups of all postnatal ages and did not appear to change with time. Little or no vasculature staining was noted for antibodies to the basement membrane-associated protein collagen IV19 or to the endothelial cell–related proteins transferrin20 and prostaglandin F2α,21 the extracellular matrix protein fibronectin,22 or the pericyte marker desmin.23 Confocal microscopy was employed to quantify the intensity of staining for antibodies to laminin and collagen V and to obtain high-resolution images, as shown in Figure 3. These data are found in Table 3 and demonstrate a significant increase in both extracellular matrix components at PND 4 compared with PND 1. By PND 10, the intensity measurements for both are less than those at PND 4; antibody staining for collagen V at PND 10 is also less than that at PND 1.

In contrast, when microvessels of the periventricular white matter were examined for the same three postnatal ages, there were no differences in signal intensity for either laminin or collagen V.

In addition, with laminin antibody–staining there appeared to be two classes of microvessels present at PND 10. One type appeared “string-like”; the second had the appearance of normal vasculature, similar to that found in all other cerebral regions studied. Both classes of vessels were stained by Bandeiraea lectin. Employing confocal microscopy on laminin antibody–stained tissues, the intensity of signals for the large vessels were more than double that for the small ones, suggesting that the small vessels had lower laminin concentrations and may represent either regressing or developing vessels.

Discussion

The capillaries of the highly vascular germinal matrix are believed to be the site of primary cerebral hemorrhage in preterm neonates. Although neuropathologic studies of preterm infants are technically difficult to perform, two earlier studies24–25 of human fetal germinal matrix through 26 weeks' gestational age suggested increasing development of glia surrounding the vasculature, decreasing amounts of extracellular space, and increasing maturity of the basement membranes. The authors could not, however, agree on the timing of “maturity” of the vessels or the gestational age at which the germinal matrix vessels became structurally similar to cortical vessels of the same gestational age. Grunnet26 examined human germinal matrix tissues from 19 through 32 weeks’ gestational age and reported that the germinal matrix vessel and lumen areas were significantly greater than those of cortical vessels of the same gestational age. She hypothesized that the greater diameter of germinal matrix vessels permitted greater pressure on their walls and thus hemorrhage.

The newborn beagle pup has been used for many years as a model for neonatal GMH/IVH.7–8 In this animal the germinal matrix is a low-flow zone that experiences marked changes in blood flow in models for hemorrhage.7,8 GMH/IVH may be readily produced within the first 48–72 postnatal hours by the clinically relevant models of acute hypercarbia, acute hypertension, and hemorrhagic hypotension followed.
TABLE 2. Germinal Matrix Vessel Antibody Staining in Beagle Pups of Three Postnatal Ages

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Postnatal day</th>
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<tbody>
<tr>
<td>α-Smooth muscle actin</td>
<td>−</td>
</tr>
<tr>
<td>α2-Macroglobulin</td>
<td>+</td>
</tr>
<tr>
<td>Collagen V</td>
<td>++</td>
</tr>
<tr>
<td>Endothelin</td>
<td>+</td>
</tr>
<tr>
<td>Factor VIII-related antigen</td>
<td>+</td>
</tr>
<tr>
<td>Glial fibrillary acidic protein</td>
<td>+</td>
</tr>
<tr>
<td>Laminin</td>
<td>+++</td>
</tr>
<tr>
<td>Vimentin</td>
<td>+</td>
</tr>
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<td>++</td>
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</table>

Values are relative staining: −, +, ++, or +++.

by volume re-expansion. A retrospective review of the first 25 litters of animals exposed to a hemorrhagic hypotension/volume re-expansion model for GMH/IVH in our laboratory revealed an incidence of 73% GMH/IVH on PND 1, 45% on PND 2, and 25% on PND 3.8 In our studies, the incidence of hemorrhage in noninsulted pups has been 15–20%. Trommer et al33 performed light and electron microscopy on the brains of two newborn beagle pups and demonstrated that the germinal matrix vessel density was similar to that of the white matter but less than that of the cortex. The mean germinal matrix vessel diameter was reported to be slightly larger than that of the cortex, caudate, or white matter. In addition, the germinal matrix vessels were reported to be thinner along greater portions of their circumferences than vessels from other regions. Trommer et al33 hypothesized that the larger size, thinner walls, and diminished support from the surrounding neuropil increased these vessels' risk for hemorrhage.

Leuschen et al34 performed serial neuropathologic studies of newborn beagle pup brains at 24, 48, and 72 hours' postnatal age. Cortical vessels were compared with germinal matrix vessels. All vessels studied had junctional complexes, and there were no differences across postnatal ages in the number or distribution of functional complexes of the germinal matrix vessels. In contrast, lumina of the germinal matrix vessels were significantly larger than those of the cortical vessels. In addition, a significant proportion of the germinal matrix vessels with large lumina

FIGURE 2. Photomicrographs of immunofluorescent staining for antibodies to laminin in germinal matrix microvessels of beagle pups at postnatal days (PNDs) 1 (a), 4 (b), and 10 (c) demonstrate that antibodies to laminin were present on PND 1 and increased markedly by PND 4. At PND 10, vessel density is decreased although laminin staining appears unchanged from that at PND 4. Bar=250 μM.

FIGURE 3. Confocal microscopy images of germinal matrix microvessels of beagle pups stained with antibodies to laminin at postnatal days (PNDs) 1 (a), 4 (b), and 10 (c) demonstrate significant increase in laminin at PND 4 compared with PND 1. At PND 10 note appearance of two sizes of laminin-staining structures, the larger (arrowhead) representing typical blood vessels and the smaller (arrow) likely representing developing or regressing vessels. Bar=50 μm.
underwent progressive attrition with time. These data thus demonstrated active modification of the germinal matrix vessels of the newborn beagle pup during the first several postnatal days.

Our current studies suggest that the germinal matrix microvessels of the newborn beagle pup undergo changes in both structure and density during the first 10 PNDs. We chose animals of ages 1, 4, and 10 PNDs. These times include the peak (PND 1) and end point (PND 4) of the risk period for GMH/IVH in this model, as well as a time well outside this interval. Bandeiraea lectin stains for endothelial cells were employed to determine microvessel density. These data demonstrated no difference in vessel density during the first 4 PNDs—the presumed risk period for GMH/IVH in this model. By PND 10, however, there was a significant decrease in germinal matrix vessel density. No change was noted in periventricular white matter vessel density across postnatal ages, although cortical vessel density also decreased at PND 10.

Gliarial fibrillary acidic protein–positive cells believed to be astrocytes have been detected as early as 16 weeks' gestational age in the developing human brain; we found little difference in germinal matrix vascular staining for vimentin and glial fibrillary acidic protein, two constituents of cerebral astrocyte intermediate filaments, during the time interval studied. In contrast, we failed to find evidence for α-smooth muscle actin in germinal matrix vessels until PND 10. Actin has been reported to localize to the microfilament bundles of pericytes and is believed to be associated with contractile properties of this cell type. We cannot rule out the possibility that the tissue preparation, particularly the fixation, is responsible for the absence of staining for α-smooth muscle actin until PND 10. Uniform tissue preparation among animals of all age groups permits us to conclude, however, that the α-smooth muscle actin content increases in germinal matrix microvessels between PND 4 and PND 10.

In addition, although there were no differences in some endothelial cell–related antibodies (including those to α2–macroglobulin, endothelin, and factor VIII–related antigen) during this time interval, we noted time-related changes in antibody staining for laminin and collagen V. By PND 4, an age at which newborn beagle pups are believed to be relatively resistant to GMH/IVH, a significant increase in both matrix proteins was found. The later decrease in total field fluorescence attributable to laminin and collagen V found at PND 10 may reflect the lower vessel density in the germinal matrix at this postnatal age. The uniform treatment of the tissue among animals and age groups should make the developmental comparisons presented here valid. In addition, Z-plane sectioning using confocal microscopy allowed us to examine standardized optical "cuts" through tissue sections, thus permitting reasonable comparison of a series of slides.

Laminin is a major constituent of vascular basement membranes and is thought to be an early indicator of vascular maturation. The basement membrane is a selective barrier that strengthens vessel walls; it is synthesized by the cells that rest on it and is composed of collagen and other specific molecules that function as structural elements as well as substrates for cell adhesion. Laminin is a large cross-shaped glycoprotein composed of three disulfide-bonded polypeptide chains and exhibits multidomain structural and functional units that enable it to bind to itself, collagen IV, and heparan sulfate as well as several different types of laminin receptor proteins on the surface of the cells to which it attaches. In endothelial cell culture, at least two well-studied binding proteins codistribute with laminin on the exterior of cells and with actin filaments on the interior, suggesting transmembrane actin–laminin links.

In addition to serving as a basement membrane structural protein, laminin has also been demonstrated to influence the proliferation, migration, shape, and cytoskeletal organization of endothelial cells in culture and thus may also be involved in signal transduction in developing blood vessels. The collagens are a family of fibrous proteins with stiff, triple-stranded helical structures. Types IV and V collagen molecules are found in the basal lamina, where they assemble into a sheetlike meshwork and cross-link with other membrane proteins to provide tensile strength for vascular walls.

These studies demonstrate that there is no difference in germinal matrix vascular density between PND 1 and PND 4—the known risk period for GMH/IVH in the newborn beagle pup. During the same time there is a significant increase in the laminin concentration and a lesser increase in the collagen V concentration in the same system. We speculate that these well-recognized basement membrane proteins produce sufficient structural integrity to germinal matrix vessels to prevent capillary rupture and thus intraventricular hemorrhage.

References

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Postnatal day</th>
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<tbody>
<tr>
<td></td>
<td>1 (n=4)</td>
</tr>
<tr>
<td>Laminin</td>
<td>43.1±6.9</td>
</tr>
<tr>
<td>Collagen V</td>
<td>45.1±4.1</td>
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</table>

Values are mean±SEM pixel intensity/mm². *p<0.05 different from postnatal day 1 by Student's unpaired t-test.
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