Indomethacin Promotes Germinal Matrix Microvessel Maturation in the Newborn Beagle Pup

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Background and Purpose: Although indomethacin has been demonstrated to prevent germinal matrix and intraventricular hemorrhage in clinical and animal studies, the mechanism of action of this agent to prevent hemorrhage remains unclear. Previous studies have demonstrated both that the microvessels in the germinal matrix of newborn beagle pups undergo basement membrane maturation during the first 4 postnatal days and that indomethacin may promote laminin deposition in tumor cell culture systems.

Methods: We employed the newborn beagle pup model to test the hypothesis that indomethacin may stimulate laminin deposition in germinal matrix microvessels. Newborn pups were randomized to receive either 0.1 mg/kg/dose i.p. indomethacin or an equal volume of saline diluent. Pups received doses of study medication once a day for 1, 2, or 3 days and were studied on postnatal days 1, 2, 3, or 4. Pups were anesthetized and systemically perfused with buffered formalin; the brains were removed and prepared for immunohistochemical study.

Results: Sections stained with Bandeiraea lectin demonstrated that there was no difference in germinal matrix vessel density among the postnatal ages studied; similarly, there were no differences in vessel density between saline- and indomethacin-treated animals at any postnatal age. Quantification of germinal matrix stained intensity by confocal microscopy demonstrated significant increases in indomethacin-treated pups for both laminin staining at postnatal days 2 (p=0.05) and 3 (p=0.0009) and type V collagen staining at postnatal day 2 (p=0.011). Although staining for β integrins increased across postnatal ages, there were no differences between saline- and indomethacin-treated animals.

Conclusions: These data suggest that indomethacin may stimulate basement membrane deposition in the germinal matrix microvessels of newborn beagle pups to prevent germinal matrix and/or intraventricular hemorrhage.

KEYWORDS • indomethacin • microcirculation • newborn • dogs

Multiple studies have suggested that indomethacin, a known inhibitor of the cyclooxygenase step of prostaglandin synthesis, may prevent germinal matrix and/or intraventricular hemorrhage in preterm neonates.1-4 In animal studies, indomethacin decreases baseline cerebral blood flow and inhibits prostaglandin synthesis.5-8 In addition, in a hemorrhagic hypotension/volume reexpansion model for germinal matrix and/or intraventricular hemorrhage in the newborn beagle pup, indomethacin blunted those changes in systemic blood pressure that the insult produced but did not alter changes in cerebral blood flow.9,10 Thus, the exact mechanism of action by which this pharmacological agent may prevent germinal matrix and/or intraventricular hemorrhages remains uncertain.

Our newborn beagle pup studies have previously demonstrated a developmental increase in the basement membrane proteins laminin and type V collagen in germinal matrix microvessels during the first 4 postnatal days.11 This same time interval represents the "risk period" during which germinal matrix and/or intraventricular hemorrhages may be produced in this animal by clinically relevant experimental manipulations.9,10,12-16 Recent studies have suggested that indomethacin may induce laminin deposition in cell culture systems.17,18 We therefore employed the newborn beagle pup model to test the hypothesis that indomethacin may stimulate laminin deposition in germinal matrix microvessels. Such an increase in basement membrane protein may strengthen these vessels and thus prevent germinal matrix and/or intraventricular hemorrhages.

Materials and Methods

The following procedures and protocols were approved by the Yale University Committee for Animal Care.

Pregnant beagles were obtained 2 weeks before their expected whelping date. The date and time of birth of each litter of pups were noted, and at approximately 6 (range, 4-10) postnatal hours the pups were randomized to receive either 0.1 mg/kg/dose i.p. indomethacin...
or an equal volume of saline diluent. Pups received their first dose of study medication immediately following randomization, at approximately 6 postnatal hours, and were then returned to their dams. Pups received second and third doses of study medication 24 and 48 hours later, at approximately 30 and 54 postnatal hours, respectively. Pups killed after only two doses, at approximately 42 postnatal hours, will be referred to as postnatal day 2 (PND 2); pups that received all three doses and were killed at approximately 66 postnatal hours will be labeled PND 3. Pups that received all three doses of study medication and were killed at approximately 90 postnatal hours will be referred to as PND 4. PND 1 refers to those pups that did not receive study medication and were killed at 6 postnatal hours.

Before sacrifice, the pups (n=34) were anesthetized with 30 mg/kg pentobarbital. After they were unresponsive to deep noxious stimulation, a thoracotomy was performed and they were systemically perfused with 0.01 mg/ml in phosphate buffered formalin. The brains were removed and placed in 0.1 M phosphate buffer over-night. The following day the brains were placed in 0.1 M phosphate buffer and then incubated for 3 hours in tetrath-ylrhodamine isothiocyanate-labeled secondary antibodies (1:100 concentration, Sigma). Following this incubation, the sections were washed with phosphate buffered saline (pH 7.2) three times, mounted on glass slides, and coverslipped with DPX mountant (Fluka Chemie AG, Buchs, Switzerland). Immunohistochemical controls were tissue sections from each pup prepared as above but without any primary antibody.

Confocal microscopy was performed using an MRC-600 scanning laser microscope (Bio-Rad, Cambridge, Mass.) connected to a Zeiss standard microscope (Thornwood, N.Y.) also equipped with conventional epifluorescence illumination. The conventional system was used to identify the region of interest on each section. Confocal microscopy was performed on three sections from each pup; three determinations were made on each tissue section, and the area of each determination was 56,532 μm².

To achieve a reproducible sampling technique for data acquisition using the confocal microscope, settings that would affect laser intensity, optical section thickness, and detector sensitivity were optimized to give an acceptable signal-to-noise ratio for each antibody. These settings remained unchanged within each experimental group analyzed so that changes in pixel intensity would be representative of real labeling density. These settings included using the same neutral density filters (#3 for laminin, #2 for type V collagen), objective lens (x16), confocal aperture settings (optical section thickness), and faster-controlled zoom factor (1.5). Images thus created and stored as image files on the host computer’s optical disk would then possess identical “volumes” of fluorescence signal. Images were then analyzed using the MRC system software for pixel intensity and area to quantify relative changes between tissues from different animals.

Student’s t tests were used for the analysis of intensity and density data.

Results

The germinal matrix microvessel density data (Table 1) demonstrate no difference across the postnatal ages examined. Similarly, there was no difference in germinal matrix vessel density at any postnatal age for the indomethacin-treated pups compared with the saline-treated animals.

By employing routine fluorescent microscopy, germinal matrix microvessel staining for antibodies to laminin, type V collagen, and the β1 integrin chain were found to increase across postnatal ages for both the saline- and indomethacin-treated groups.

| TABLE 1. Vessel Density in Indomethacin- and Saline-Treated Pups |
|---|---|---|---|---|
| Treatment | Postnatal day | Density (mm/mm²) | n | Density (mm/mm²) | n | Density (mm/mm²) | n | Density (mm/mm²) | n |
| Saline | 1 | 8.8±3.0 | 7 | 10.4±1.3 | 5 | 9.8±2.9 | 4 | 8.6±3.8 | 4 |
| Indomethacin | 2 | 9.8±1.2 | 7 | 10.1±3.9 | 5 | 8.4±2.0 | 2 | 9.8±1.2 | 5 |

Values are mean±SEM.
FIGURE 1. Immunofluorescence micrographs of germinal matrix microvessels from newborn beagle pups treated with saline (A, C, and E) or indomethacin (B, D, and F). Sections were labeled with antibodies directed against laminin. \( \phi \), Postnatal day (PND) 1 control; A and B, PND 2; C and D, PND 3; E and F, PND 4. Scale bar=100 \( \mu \)m.

Confocal microscopy was employed to quantify the intensity of staining for antibodies to laminin and type V collagen and to obtain high-resolution images, as shown for laminin staining in Figure 1. The laminin data (Figure 2) demonstrate significant increases in this extracellular matrix component for indomethacin-treated pups compared with their saline-treated littersmates at postnatal days 2 (\( p=0.05 \)) and 3 (\( p=0.0009 \)). At postnatal day 4 there was no difference in laminin intensity between saline- and indomethacin-treated animals. Similarly, the intensity staining data for type V collagen (Figure 3) demonstrate an increase in collagen at postnatal day 2 for indomethacin-treated animals compared with saline-treated pups (\( p=0.011 \)).

Similar quantitative studies of the periventricular white matter adjacent to the germinal matrix regions studied demonstrated no difference in either immunoreactivity across postnatal ages or between the indomethacin- and saline-treated groups.

Germinal matrix microvessel \( \beta_1 \) integrin staining was not quantified by confocal microscopy because of the increasing staining by this polyclonal antibody at advancing postnatal ages of not only germinal matrix microvessels but also of the multiple other cellular types present. Fluorescent microscopic assessment by two observers who were blinded to the treatment groups suggested no difference in \( \beta_1 \) integrin staining between indomethacin- and saline-treated pups at any postnatal age.

Discussion

Shankaran et al\(^2\) have recently reported that intraventricular hemorrhage, or hemorrhage into the germinal matrix tissues of the developing brain, occurred in 45.5\% of 2,928 neonates weighing <1,500 g at birth.

FIGURE 2. Bar graph of mean±SEM relative fluorescence intensities of germinal matrix microvessels from newborn beagle pups treated with saline (open bars) or indomethacin (shaded bars). Sections were labeled with antibodies directed against laminin at postnatal days 1, 2, 3, and 4, and fluorescence intensities were quantified. \(* p=0.05; ** p=0.0009 \) different from saline.

FIGURE 3. Bar graph of mean±SEM relative fluorescence intensities of germinal matrix microvessels from newborn beagle pups treated with saline (open bars) or indomethacin (shaded bars). Sections were labeled with antibodies directed against type V collagen at postnatal days 1, 2, 3, and 4, and fluorescence intensities were quantified. \(* p=0.011 \) different from saline.
Infants with germinal matrix and/or intraventricular hemorrhages are at higher risk for periventricular leukomalacia, hemorrhagic white matter lesions, increased intracranial pressure, seizures, and hydrocephalus and are thought to be at higher risk for neurodevelopmental handicap than their peers without hemorrhage.22-25

Although intraventricular hemorrhage has been attributed to changes in cerebral blood flow to damaged germinal matrix microvessels, the pathophysiology of prevention of hemorrhage remains unclear.22 Multiple pharmacological agents including phenobarbital,26-27 ethamsylate,28 vitamin E,29,30 pancuronium,31 vitamin K,32 and indomethacin have been investigated with varying results in both animal and clinical trials.

Indomethacin has been reported to inhibit the cyclooxygenase step of prostaglandin synthesis and decrease baseline blood flow in animal studies.6-8 In the newborn beagle pup hemorrhagic hypotension/volume reexpansion model of germinal matrix and/or intraventricular hemorrhage, indomethacin decreased the incidence of insult and blunted the systemic blood pressure changes associated with it.9,10 Indomethacin did not, however, alter the changes in cerebral blood flow that insulted pups experienced.10

In newborn preterm infants indomethacin has been shown to cause decreases in cerebral blood flow velocity for up to 120 minutes.33-35 Because these changes in cerebral blood flow are reported to be associated with significant increases in systemic blood pressure and are not replicable after subsequent doses of indomethacin,25,26 clinical studies also do not support prolonged alterations in cerebral blood flow as the primary mechanism of action of indomethacin to prevent germinal matrix and/or intraventricular hemorrhages.

Similar to that in preterm neonates, the risk period for hemorrhage in newborn beagle pups is 3-4 days.9,10,12-14 We hypothesized that this risk period is related to rapid changes in the germinal matrix vasculature during the first few days after birth, and our previous studies have demonstrated that amounts of the extracellular matrix components laminin and type V collagen increase significantly during the first 4 postnatal days in germinal matrix microvessels of newborn beagle pups.11 We have speculated that these basement membrane proteins may add sufficient structural integrity to germinal matrix vessels to prevent capillary rupture and thus germinal matrix and/or intraventricular hemorrhages.

Laminin is a major constituent of vascular basement membranes; the deposition of laminin during development is thought to be an early indicator of vascular maturation.11,38-40 The basement membrane is composed of collagen and other specific molecules that function as both structural elements to provide tensile strength and substrates for cell adhesion and inducers of differentiation. Laminin is a large cross-shaped glycoprotein; it is composed of three disulfide-bonded polypeptide chains and possesses functional domains that enable it to interact with itself, nidogen, type IV collagen, and heparan sulfate as well as several different types of laminin receptor proteins.41

The integrins are a family of receptors that mediate cell–cell and cell–substratum interactions.42 Integrin binding to extracellular ligands has been reported to regulate cell adhesion and influence cell shape, motility, and gene expression.43 Many of the integrins that are associated with extracellular matrix adhesion share the common β1 subunit, whereas specificity for ligand binding is determined, at least in part, by different units.44 Laminin binding has been reported for α5β1, α6β1, and αβ integrin heterodimer pairs, and recent sequence analyses of complementary deoxyribonucleic acids encoding human laminin A and B chains have revealed the presence of several potential cell binding sites able to interact with several cell surface integrins and non-integrin-binding proteins.45-47

Cellular adhesion to laminin has also been ascribed to non-integrin-binding proteins.47 A variety of tumor cells have been shown to bind laminin,17,18,48-51 and in these cell culture systems both the amount of laminin and the level of laminin receptor expression on the cell surface have been correlated with cellular motility and induction of a type IV collagenase and thus the capacity of the cells to metastasize.17,18

When Alino and colleagues18 evaluated the effect of indomethacin on the lung metastatic potential of a low-metastatic Lewis lung carcinoma and its ability to bind laminin on the cell surface, they demonstrated that indomethacin increased both the binding of [125I]laminin on the cell surface and the metastatic rate. Scatchard analysis revealed that the incubation of these tumor cells with 10-7 M indomethacin induced a specific increase in the number of laminin binding sites on the cell surface. The metastatic effects were partially reversed by the peptides DPGYGIGSR or YIGSR, which correspond to an active site at the B1 laminin chain, with the ability to bind to a 67-kd laminin cell surface-binding protein. Alino et al18 thus suggested that indomethacin could modulate laminin cell surface receptors in this system.

The current studies demonstrate that indomethacin, administered to newborn beagle pups in clinically relevant doses, increases the immunoreactivity of the basement membrane proteins laminin and type V collagen in germinal matrix microvessels on postnatal days 2 and 3, the known risk period for germinal matrix and/or intraventricular hemorrhage in this model. The studies also confirm our previous work,11 which demonstrated a developmental increase in the immunoreactivity for these same proteins in newborn beagle pup germinal matrix microvessels. In addition, although these data also suggest a postnatal increase in staining for the B1 subunit of several possible laminin-binding integrin heterodimeric receptors, this effect does not appear to be influenced by indomethacin. Unfortunately, because of the failure of cross-reactivity of available antibodies with canine tissue, we were unable to obtain specific antibodies to those subunits of the integrin family that would permit analyses of specific laminin cell surface–binding receptors.

These data indicate that one mechanism by which indomethacin may prevent neonatal germinal matrix and/or intraventricular hemorrhages may be to stimulate laminin and type V collagen deposition in germinal matrix microvessels. The increased laminin and type V collagen deposition induced by indomethacin in these animals may be secondary to increased laminin and type V collagen synthesis by endothelial cells, increased laminin binding to cell surface membranes, or de-
creased degradation of these two extracellular matrix proteins. Since both laminin and type V collagen are multichain molecules, there is also the possibility that indomethacin modulates laminin A and B chain ratios and/or type V collagen α1, α2, and α3 chain ratios. Further molecular studies will be required to differentiate among these alternatives.

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The study by Ment et al provides the first glimpse into a potential mechanism by which indomethacin may prevent germinal matrix and intraventricular hemorrhage in newborns. The authors found that indomethacin may promote the deposition of basement membranes in microvessels in the germinal matrix during the period when the animals were vulnerable to hemorrhage. The precise mechanism by which indomethacin has this effect is not yet understood. Several obvious possibilities need to be investigated; the protective effect may be the result of inhibition of prostaglandin synthesis or inhibition of the generation of oxygen radicals, which are known to be produced by cyclooxygenase under suitable conditions. Because other antioxidants such as superoxide dismutase or vitamin E have been reported to have protective effects similar to that of indomethacin on germinal matrix and intraventricular hemorrhage, the participation of oxidants is suggested. Hydroxyl radicals attack a variety of tissue constituents including components of the extracellular matrix. It is expected that future studies will explore these mechanisms further.

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