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Temporal Expression Pattern of Cerebrovascular Endothelial Cell Alkaline Phosphatase During Human Gestation

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Abstract. In premature human neonates, immaturity of cerebral vessels can contribute to clinical problems such as germinal matrix hemorrhage and white matter damage. Afferent cerebral vessels in the brain of term babies express alkaline phosphatase (AP), an ectoenzyme located on the surface of endothelial cells. Using AP enzyme histochemistry we have examined the cerebrovasculature of premature live-born human neonates to determine when cerebral afferent vessels begin to express AP. Brains were collected at autopsy and processed for histological examination. AP-stained vessel density in the periventricular white matter was quantified using digital imaging and automated morphometry. Babies born prior to 28 wk gestation display few AP-positive vessels in the periventricular white matter, whereas, babies born after 28 wk gestation exhibit an AP-positive vascular pattern that resembles the adult pattern. In contrast, immunostaining for collagen revealed an extensive vascular network in both early and late gestation infants. Our measurements indicate that neonates born prior to 28 wk gestation are characterized by immature cerebral white matter afferent vessels and raise the possibility that the immaturity compromises vascular function.

Key Words: Alkaline phosphatase; Brain; Collagen; Neonate; Prematurity; Vessels.

INTRODUCTION

Embryonic development of the human telencephalic vasculature is related to important and serious clinical problems such as germinal matrix/intraventricular hemorrhage and white matter damage, both of which are common in premature neonates (1–3).

Cerebral blood vessel formation begins early in human gestation (4–6). By 21 days a network of vessels derived from angioblasts surrounds the neural tube. All intraparenchymal vessels are derived from this original network, which by the fifth gestational week develops sprouts. These sprouts cross the pia and the outer limiting membrane to enter the substance of the developing cerebrum where they elongate towards the lateral ventricle. These vessels, commonly referred to as transcerebral channels, extend branches that join with branches of nearby channels to form a complex network (7–9). These channels, with walls consisting of only a single layer of endothelial cells, differentiate into either arterioles or veins, or regress if their function is superfluous, while their branches form the capillary network (10). An extensive capillary network is in place prior to the end of the second trimester. The pattern of centripetal growth, with vessels growing from the brain surface towards the lateral ventricle, is followed by a period of vessel maturation that also exhibits a centripetal pattern.

The maturation period is presently rather vaguely defined. It includes the transient expression of molecules related to endothelial proliferation, the association of smooth muscle cells with maturing arterioles, as well as the accumulation of matrix molecules around all classes of vessels (11–13). In addition, arterioles and capillaries in the brains of adults and term babies are characterized by comparatively high levels of alkaline phosphatase (AP) activity (14–16), but the initial appearance of this enzyme activity during development has not been established. The present study investigates the developmental expression of AP in cerebral vessels.

The AP activity of brain arterioles and capillaries is concentrated on the surfaces of endothelial cells and, as such, is part of a large family of ecto-ATPases (17). Ecto-ATPases have garnered considerable interest because of their location, expression pattern, and association with cellular behaviors such as differentiation, transformation, and response to toxic injury (18). Though the function(s) of the enzyme activity is not clearly defined, it is possible that enzymatic products could modulate vascular tonicity, inflammatory reactions, or other physiological events important to the continued normal development and function of the cerebral tissue.

To determine the expression pattern of AP, we have made use of an enzyme histochemical technique developed and used to identify in tissue sections the arterioles and capillaries that express this enzyme (19–22). This method has been used in adult human brain to reveal the normal morphometry of these vascular elements in cerebral hemispheres (23, 24) and to reveal vascular alterations associated with normal aging, as well as with various pathological conditions such as Alzheimer disease (22, 23, 25), hypertension (24), and leukoariosis (26, 27).

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TABLE
Case Presentation

Case Number	Gestation (weeks)	Survival (days)	Adjusted age (weeks)	Birth weight (grams)	Brain weight (grams)
1	22–23	2.5	23	435	50
2	24	0.5	24	795	93
3	24	0.5	24	660	52
4	24	5	25	535	64
5	24–25	4	25	500	91
6	25	1.5	25	760	95.7
7	26	12	28	995	122
8	28	0.5	28	454	88
9	28	22	31	960	150
10	38	4	38.5	2870	330
11	36	1	36	3500	298
12	40	4	40.5	3800	440
13	40	20	43	3300	420

The technique has also played an important role in establishing the involvement of lipid emboli in producing the neural impairment that can follow cardiac surgery involving cardio-pulmonary bypass (28, 29). Moody and colleagues (15) have demonstrated that the alkaline phosphatase histochemical technique also localizes AP activity in brain tissue from premature human neonates. They then extended this application by demonstrating that germinal matrix hemorrhage in premature neonates is correlated more closely with ruptured veins rather than with ruptured arterioles or capillaries (16).

Our results indicate that the expression pattern of AP common to the brain of term babies and adults is not established until after 28 wk gestation. We discuss the possibility that the paucity of AP in the younger group may be one factor, of many, that contributes to the high incidence of neuropathology in this group.

MATERIALS AND METHODS

Brains from 18 premature neonates obtained at autopsy were studied histologically; 13 of these were used for quantitative studies. Gestational ages ranged from 23 wk to 40 wk and postnatal survival times ranged from less than 1 h to 22 days. These data and additional information pertaining to the 13 specimens used for quantitative purposes are summarized in the Table.

As soon as possible following autopsy, the neonatal brains were placed in cold 70% alcohol for 10 to 12 days for fixation (16). Subsequently, a whole-brain slice approximately 1-cm thick, incorporating both hemispheres, was obtained from each brain at and posterior to the foramen of Monro and included the thickest mass of germinal matrix as well as basal ganglia, thalamus, centrum semiovale, and cortex. The brain slices were dehydrated in ascending grades of ethanol, embedded in celloidin, and serially sectioned at 100 μm on a base sledge microtome. The 100-μm-thick sections were stained for native endothelial alkaline phosphatase by Bell and Scarrow's modification (23) of the Gomori method (30) or were stained by immunochemistry for collagen type IV. The collagen antibody (Chemicon, Temecula, CA) was a mouse monoclonal raised against human amniotic type IV collagen. Sections were incubated overnight in the antibody (1:30,000 in 2% normal goat serum) prior to incubation in biotinylated anti-mouse IgG. Antibodies were then visualized using streptavidin HRP and diaminobenzidine/H_2O_2 (DAB kit; Vector Labs, Burlingame, CA) following manufacturer's instructions. Control experiments were performed by substituting mouse ascites fluid for primary antibody. In all cases, control tissue yielded minimal background staining.

Vessel Density Measurements

While 18 cases were used for histological examination, only 13 of these were suitable technically for digital analysis of vessel density. Brains were divided into 2 groups based on gestational age at birth: early gestation ($<$28 wk; n = 7) and late gestation ($\geq$28 wk; n = 6). The average mean gestational age for the younger group was 24.3 $\pm$ 0.6 (range 23 to 26 wk), and for the older group 35 $\pm$ 2.3 (range 28 to 40 wk). Two to 3 measurements were made in the periventricular white matter adjacent to the germinal matrix of each hemisphere and used for evaluation of vessel density. Both left and right hemispheres were available for imaging and analysis in 4 of 7 in the early gestation group and 2 of 6 in the late gestation group. Left and right hemispheres were not significantly different (paired *t*-test, $p < 0.8913$, n = 12) and measurements for both hemispheres were pooled for these cases. For the remaining brains, only 1 hemisphere was available.

Collagen-stained and alkaline phosphatase-stained sections were used to determine the density of vessels in the periventricular white matter. Matching sections were cut from the same tissue block but were not necessarily adjacent. For measurement purposes, digital images of the region of interest were captured under brightfield illumination at 10× magnification using a Nikon Eclipse E600 microscope and a SPOT RT color digital camera (Diagnostic Instruments Inc., Sterling Heights, MI). Original digital images were 1,600 × 1,200 pixels, 72 pixels/

inch, and 24-bit RGB, and were analyzed with Image Processing Toolkit™ plug-in functions (Reindeer Games, Inc.) in Photoshop™ 6.0 (Adobe Systems Inc., San Jose, CA).

The digital image was processed as follows: 1) optimize tonal range and color balance, 2) filter intensity component of image and convert to grayscale, discarding color information, threshold image to binary (black/white) using the Johannsen (31) algorithm. After thresholding, the stained vessels appeared black and the background appeared white. The following measurements were made from the binary image: area fraction (% black pixels in the region of interest [ROI]), area of ROI (mm^2), and length of vessels in binary. Subsequently, the thresholded vessels were skeletonized to a single pixel width and the length/region of interest measured at this level. In our results, vessel density was expressed as length/area (mm/mm^2) of the skeletonized vessels. While the other measurements provided additional information relevant to the volume fraction of the vascular components at different gestational ages, the least amount of variability was contained in the skeletonized length/area data. Skeletonization omitted most variation in the binary conversion, due to differences in stain or background of the original slides. The length/area of collagen IV-stained versus alkaline phosphatase-stained vessels were compared for the 2 gestational age groups. Data calculation and statistical analyses were done with Excel (Microsoft Corp.) and GraphPad Prism (GraphPad Software, Inc.).

RESULTS

Immunochemistry and Enzyme Histochemistry of Cerebral Vessels

All vessels, arterioles, capillaries, and veins are presumed to be recognized by the collagen antibody (Figs. 1, 2). This method, therefore, reveals the developmental state of the entire vascular network. Babies as young as 24 wk gestation are seen to have a rich network of collagen-stained vessels in the cortex and throughout the white matter of the cerebral hemispheres. A parallel array of vessels penetrates the cortical layer and continues into the underlying white matter where longitudinal vascular profiles sweep towards the outer border of the lateral ventricle. In both the cortex and white matter, side branches of these vessels connect with capillaries lying in the spaces between the long profiles. The general morphological pattern of vessel distribution is similar at all stages examined (Figs. 1A, C, E, 2A, C). Figure 2 demonstrates a specimen from a baby born at 28 wk gestation that survived for 3 wk (Fig. 2A, B) and another born at 34 wk gestation (Fig. 2C, D). Collagen staining in both of these babies reveals a white matter vascular network considerably more complex than that seen in the younger specimens. Both babies resemble closely the vascular complexity observed in term babies. These examples would indicate that by 28 to 34 wk gestation, a mature vascular pattern has emerged.

The AP staining technique, which stains arterioles and capillaries but not veins, yields a picture in the younger neonates that differs radically from the collagen-stained specimens. In the youngest specimens, AP staining reveals a regular array of parallel channels passing through the cortical layer (Fig. 1B). Seldom are these channels seen to continue into the underlying white matter, a distinct contrast from the image seen in collagen-stained specimens of the same age where vessels pass through the cortex and into the subjacent white matter. The AP stain present in the cortical vessels ends rather abruptly at the cortical/white matter boundary as if the enzyme activity is not yet turned on. In the 24 week gestation neonate, the white matter contains few profiles of AP-stained vessels in comparison to the overlying cortex. In addition, the appearance of the few AP-stained white matter vessels differs from the appearance of collagen-stained white matter vessels. The AP vessels, rather than appearing as an array of parallel channels, instead, are seen as short fragments lying in relative isolation. At 24 wk gestation this description extends from the subcortical white matter to the periventricular zone. By week 26 of gestation, however, the picture has begun to change. More AP-positive profiles are present in the subcortical white matter (Fig. 1D). In the deep white matter there are still relatively few parallel vessels but there are more AP-positive fragments of vessels. In specimens from babies born at 28 wk gestation or later, AP-positive vessels are a prominent feature of the periventricular, deep white matter (Figs. 1F, 2B, D). It is evident upon visual inspection that, with increasing gestational age, a dramatic increase in deep white matter AP-staining has occurred. At gestational week 28, straight channels pointing towards the lateral ventricle are observed as well as side branches connecting these straight channels to capillary beds.

The AP staining pattern revealed by our histochemical evaluation indicates that younger specimens lack AP enzyme activity on the majority of vessels in the deep white matter. This qualitative impression was tested by quantification of the density of AP-stained vessels in the deep white matter of the younger versus the older groups of neonates.

Quantitative Measures of Vessels in the Deep White Matter

To quantify the developmental appearance of AP in the deep white matter, AP-stained vessels were measured using digital image analysis and morphometry. Figure 3 shows the image transformation process used to obtain quantifiable data for collagen-stained and AP-stained sections of brains, one from a baby born at 24 wk gestation (Fig. 3A, B), the other from a baby born at 28 wk gestation (Fig. 3C, D). Digital capture of the light microscopic images, such as those shown in Figure 3A1–D1, were processed into binary figures (Fig. 3A2–D2), which in turn were skeletonized (Fig. 3A3–D3), a process that

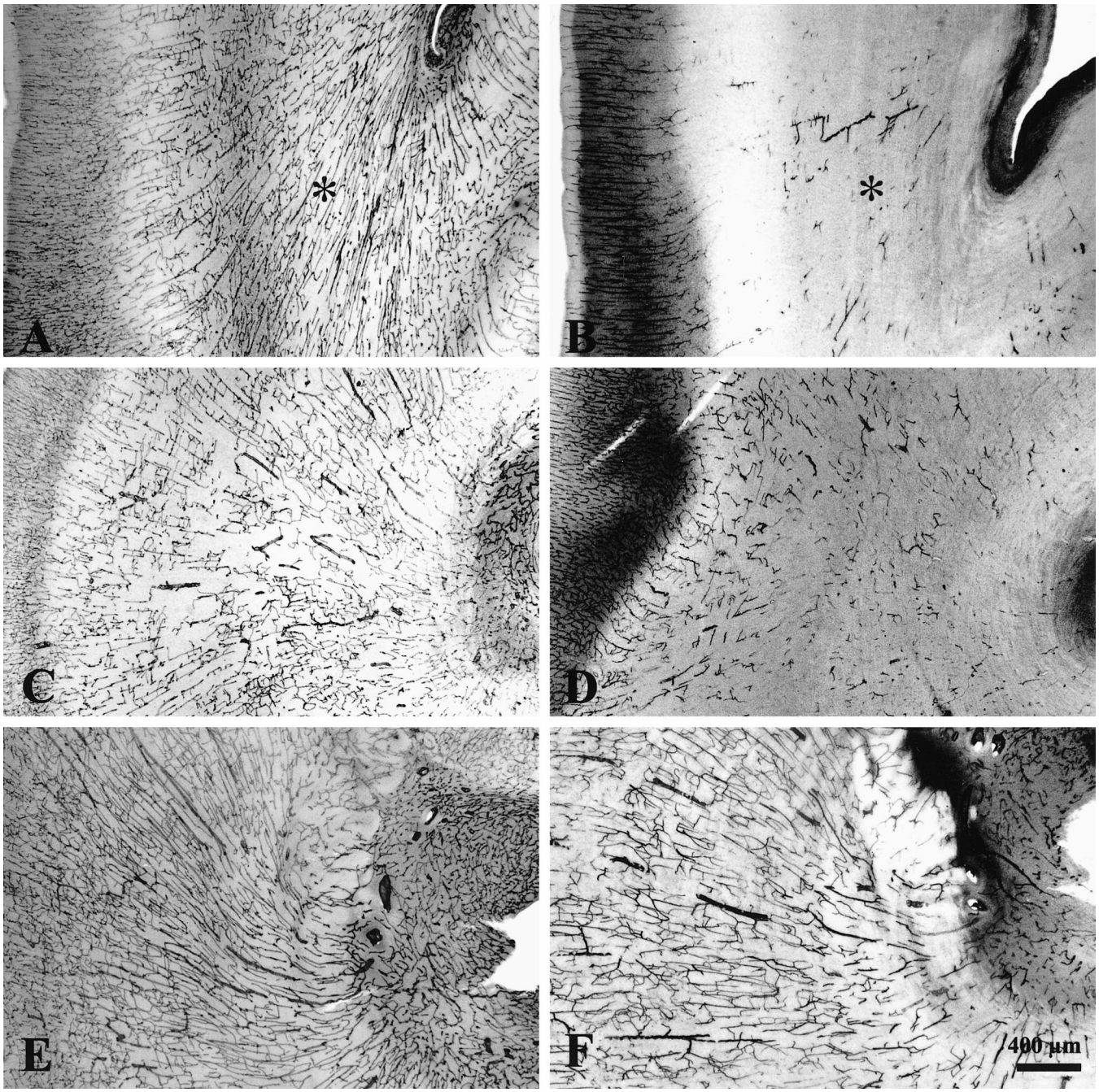

Fig. 1. Collagen immunostaining is shown in panels (A), (C), and (E). Alkaline phosphatase (AP) histochemical staining is shown in panels (B), (D), and (F). A, B: Twenty-four weeks gestation. C, D: Twenty-six weeks gestation. E, F: Twenty-eight weeks gestation. Collagen immunostaining reveals a network of vessels at all ages, whereas AP staining is low at 24 wk gestation and increases by 28 wk gestation.

creates a 1-pixel-wide midline trace of each vessel present in the binary image. A qualitative measure of the precision of the transformation process can be made by visual comparison of the 3 figures across each of the 4 rows of Figure 3. The graph in Figure 4 presents the vessel length/area measurements of collagen-stained versus AP-stained vessels in the white matter of the 2 neonatal groups. For the young group, the mean gestational age at birth was 24.3 wk with a range of 23 to 26 wk; for the older group the mean gestational age was 35 wk with a range of 28 to 40 wk. In the young group of neonates, collagen-stained vessels in the deep white matter, equivalent to areas shown in Figure 3, measured 14.4 mm/mm^2. This figure increased to 21.0 mm/mm^2 in the older group. For AP staining, which was quantified in the same 2 groups of neonates, the younger group measured

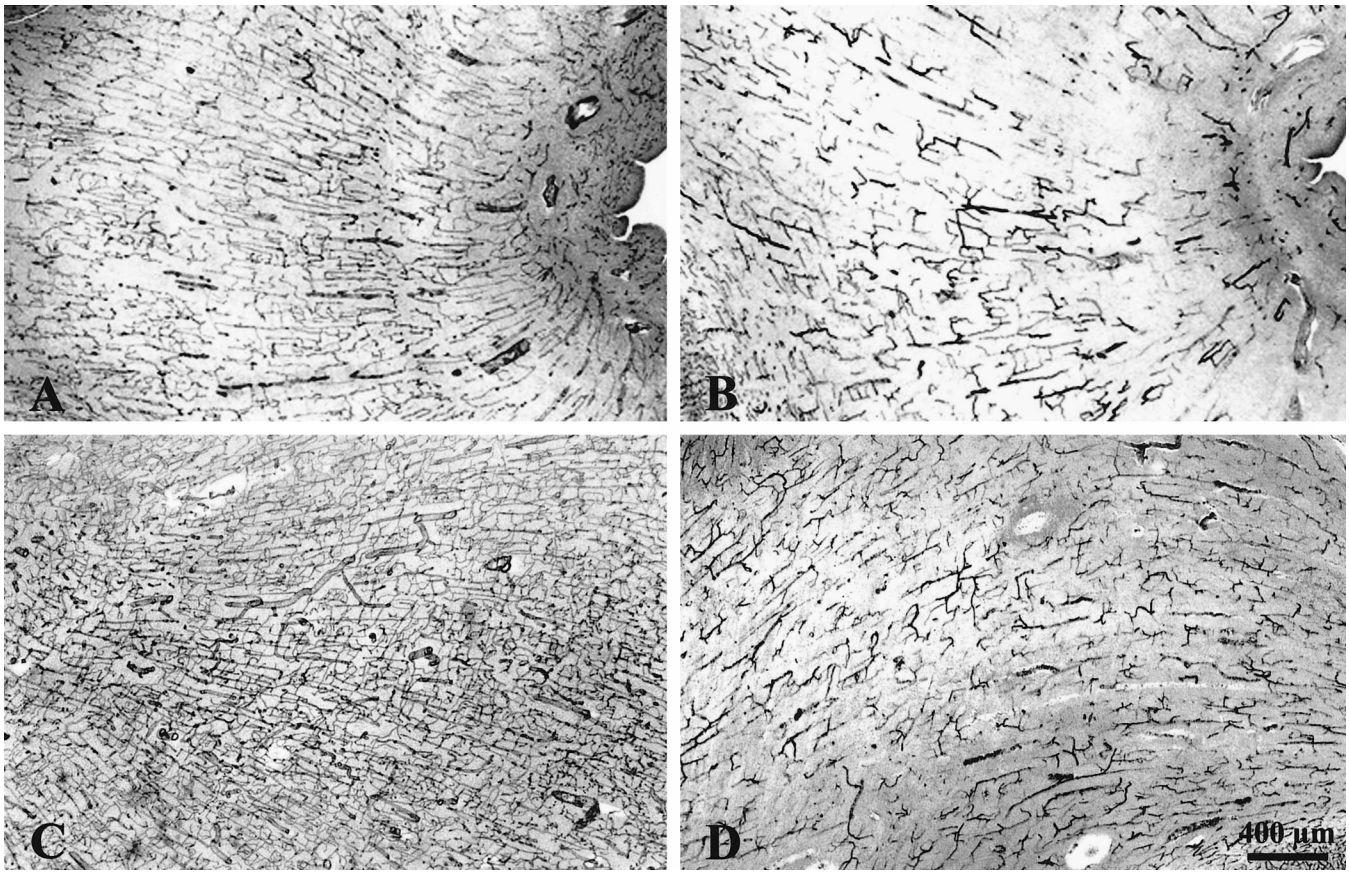

Fig. 2. Collagen immunostaining is shown in panels (A) and (C). AP histochemical staining is shown in panels (B) and (D). A, B: Twenty-eight weeks gestation and surviving for 3 wk. C, D: Thirty-four weeks gestation. Both collagen staining and AP staining reveal a dense vascular network.

2.9 mm/mm^2. This number increased to 10.4 mm/mm^2 in the older group of neonates. Whereas the collagen staining increased by 45.8% in the older group versus the younger group, the AP staining increased by 257.5% in the older group compared to the younger group.

One-way analysis of variance of the 4 groups (young AP, young collagen, old AP, old collagen) showed that the groups were significantly different ($p < 0.0001$). Post-hoc comparisons of the treatment groups with the Newman-Keuls multiple comparison test revealed highly significant differences between the vascular length/area of young and old collagen- and alkaline phosphatase-labeled vessels.

DISCUSSION

The results of our study indicate that arterioles and capillaries in the deep white matter of our young group of preterm babies are not fully mature. These vessels lack the AP activity that characterizes afferent brain vessels in humans that have developed to at least 35 wk gestation, which is the mean gestational age of our older group of preterm babies. Microscopic observation of each of the individual specimens in both groups indicates that by 28 wk gestation, the AP pattern of cerebral vessels in the deep white matter resembles that seen in term babies. Prior to 28 wk gestation, the pattern appears immature. According to current models of pathogenesis (32–36), white matter damage in premature neonates results from a combination of insults and developmental immaturity. It is possible that the sparse amounts of AP in cerebral white matter arterioles and capillaries in the youngest babies is a reflection of a vascular developmental immaturity that renders white matter in these babies particularly susceptible to insults.

Evidence that White Matter Capillaries May Be Immature

It is widely held that vessels within the germinal matrix of premature neonates are structurally immature (37–42). Arterioles in the white matter of these babies also are still under construction since they have not yet acquired a smooth muscle layer and, in fact, the smooth muscle layer of extrastriatal arterioles is not completed until after term (10). Despite this indication that the arterioles are immature, there was little evidence to indicate

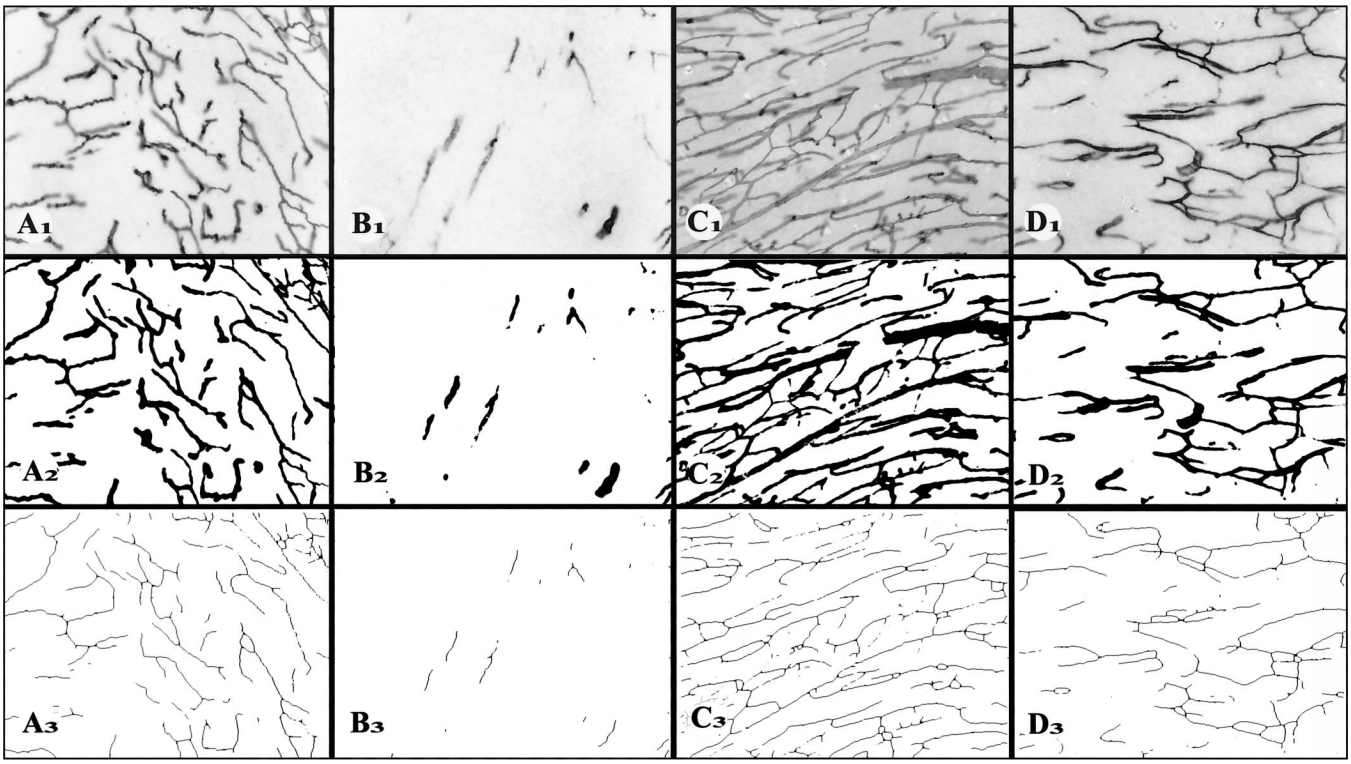

Fig. 3. Digital imaging of collagen-stained and AP-stained vessels in the deep white matter of premature neonates. A, B: Sections from a neonate born at 24 wk gestation. C, D: Sections from a neonate born at 28 wk gestation. A, C: Collagen immunostaining. B, D: AP histochemical staining. A_1–D_1, digital image of vessels in the deep white matter. A_2–D_2, images obtained by translating A_1-D_1 into binary images. A_3–D_3, skeletonized images of A_2–D_2. The skeletonized images were used to measure the length of stained vessels per area. By comparing the skeletonized image to the binary image and the binary image to the digital photomicrograph, the accuracy of the measurements is revealed.

that capillaries in the white matter were immature. However, in addition to our data indicating this immaturity, other information could be interpreted similarly. Arai et al (11) reported on the localization of vascular endothelial growth factor (VEGF) in the brain of premature human neonates. In the deep white matter, VEGF was expressed at 17 wk gestation but not prior to this stage. The concentration of VEGF in the white matter increased from 17 wk and was highest between 24 and 28 wk gestation. After this period, the concentration decreased gradually. Since the presence of VEGF indicates that vascular development is in progress, this data indicates that vessels, including capillaries in the deep white matter of fetuses between 17 and 28 wk gestation, are actively engaged in the formation of new vessels. As angiogenesis is a progressive process, taking some time to complete, it is quite likely that many of the deep white matter vessels are not fully mature during this gestational interval.

In another study, Kamei et al (12) examined vessels of neonatal brain to determine the expression pattern of collagen VI, a collagen variant thought to bind basal laminae to large structures such as neurovascular bundles (43). Collagen VI antibodies recognized leptomeningeal vessels from the earliest stages of gestation. However, it was not until 21 wk gestation that the antibodies recognized vessels in the deep white matter and it was at 38 wk gestation before they recognized vessels in the superficial white matter and the cortex. These data indicate that vessels in the cerebral white matter, as well as in other regions, are undergoing structural maturation up to and perhaps beyond 38 wk gestation.

Mechanism of AP Accumulation

The number of AP-stained vessels in the white matter increases markedly during the gestational interval between 24 wk and 28 wk. Two mechanisms that could account for this increase are readily apparent. The increase could be the result of newly expressed or enzymatically activated AP in pre-existing vessels that were originally AP-negative. Alternatively, the increase in AP-positive vessels could be due to the growth of new AP-positive vessels into the white matter. New vessels, in fact, are growing into the white matter throughout this interval, as has been noted by Kuban and Gilles (10), and as indicated in this report by the increase in the density of collagen IV-labeled vessels during the 24- to 28-week gestational interval. However, the increase in vessel density as expressed by the increase in collagen-stained vessels appears to be due

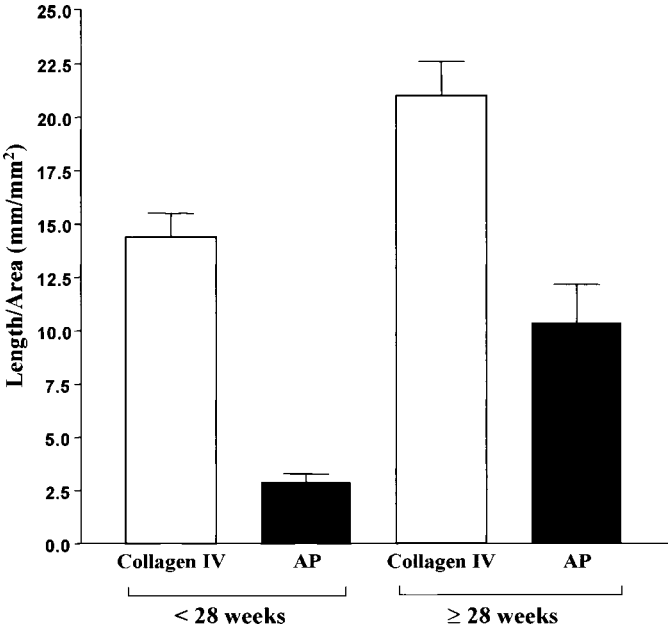

Fig. 4. Quantification of white matter staining in 2 groups of neonates. Neonates born before 28 wk gestation are compared to neonates born at or after 28 wk gestation. The concentration of collagen-stained vessels increases by approximately 45% in the older group versus the younger group. The concentration of AP-stained vessels increases by more than 250%.

largely to an increase in the formation of capillaries, whereas the increase in AP-positive vessels is due both to an increase in the number of stained capillaries as well as to an increase in the number of stained transcerebral vessels. We believe the large transcerebral vessels that come to express AP by gestational week 28, most likely appear as a result of the expression of AP in a pre-existing AP-negative vessel. It should be noted, however, that the increase in the density of AP-positive vessels in our young group of babies (with a median age of 24.3 wk gestation) versus our older group of babies (with a median age of 35 wk gestation), is nearly identical to the increase in collagen-positive vessels displayed by these 2 groups during this time period.

Risau et al (44) studied the expression of AP in the developing brains of mice, chicks, and quail and determined in each of these animals that the growth into the brain of vessels, identified by endothelial-specific markers such as Factor VIII, preceded the appearance of endothelial AP. These authors also noted that the appearance of AP in mice, chicks and quail followed a characteristic spatial pattern beginning at the brain periphery and proceeding towards the center of the brain. This pattern resembles what we observed in the human brain where AP was seen in the cortex of the youngest specimens while being relatively rare in the deep white matter. Indeed, Norman and O'Kusky (14), in a study of cortical vessel development, noted that AP could be identified as early as the fifteenth gestational week. Though they were not concerned with AP expression in the white matter, it is evident from their photomicrographs that, whereas AP is present in cortical vessels of the younger specimens, it is scarce in the white matter immediately subjacent to the cortex.

Risau et al (44) proposed that differentiation of brain-specific vascular features such as AP expression is stimulated by surrounding neural tissue, noting, for example, that non-brain vessels growing into transplanted brain tissue do, in fact, develop brain-specific features such as a blood-brain barrier (BBB) (45). Our data are consistent with the interpretation that vessel growth is initially independent of specialization (i.e. AP expression). Most likely, in humans, vessels growing into the brain from the perineural network are initially AP-negative. The developing cortex then acquires, by at least the fifteenth gestational week, the ability to stimulate AP expression in vessels passing through this region. Later, around the twenty-sixth gestational week, the underlying white matter similarly acquires the capacity to influence the properties of the existing vessels resulting in the expression of AP in the white matter vessels.

AP and the Blood-Brain Barrier

Risau et al (44) pointed out that the expression of several histochemical and immunochemical markers, including AP, correlated with the appearance of a functional BBB in mice, chicks, and quail. Though these markers are not responsible for the creation of the barrier, Risau et al (44) concluded that the BBB in these organisms appeared towards the end of the gestational period, prior to birth. If, in the case of humans, AP similarly appears coincident with the onset of BBB function, our data would indicate that the human BBB begins to function in the deep white matter at approximately 26 to 28 wk gestation, corresponding to the developmental stage when significant levels of AP are detected in white matter vessels. This estimate corresponds well with data derived from physiological studies of BBB function. Adinolfi (46) reviews physiological studies related to BBB development and concludes that in the human, BBB function is detected at approximately 27 wk gestation. Before this stage, serum proteins pass freely from the blood serum into the cerebrospinal fluid and the extracellular space of the brain. In light of these previous studies, especially that of Risau et al (44) where the temporal expression of AP is considered coincident with the appearance of a functional BBB, our data, showing a paucity of AP in white matter vessels prior to 28 wk gestation, re-emphasizes the point that babies born prior to this stage lack a functional BBB in the white matter. Thus, any substance ingested by, or injected into, these neonates will gain access to the extracellular environment of the white matter

where it could influence the ongoing cellular developmental events.

The maternal/placental deprivation hypothesis (32) postulates that the fetus benefits from factors provided by the mother or placenta until a time when the fetus can provide these factors for itself. For babies born prematurely, the benefit of maternal/placental-derived factors is lost before these babies are sufficiently developed themselves to make up this loss. While this deprivation may affect all organ systems, the brain appears particularly susceptible. It has been hypothesized that white matter damage may be one consequence of maternal/placental deprivation of growth factors (34). If the products of AP do play an important role in the fetal brain, it is possible that the role is fulfilled by maternal/placental-derived factors until late in gestation. Alternatively, a role for AP may not commence until birth. In either case, premature birth during the end of the second trimester or early in the third trimester would result in a brain incapable of producing adequate amounts of AP-derived products. The lack of an AP-derived substance could result in the tissue being unable to carry out normal regulatory functions related to, for example, blood flow or inflammation. The result could be tissue injury culminating with white matter damage sufficient to produce clinical symptoms.

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