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Expression in the Placenta of Neuronal Markers for Perinatal Brain Damage

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ABSTRACT

Determination of S-100 a and b and neuron-specific enolase (NSE) in (cord) blood and amniotic fluid has been used to assess neonatal neuronal damage after compromising conditions. However, these proteins are not only found in nervous tissue, and their expression in placenta and umbilical cord has never been investigated. In this study, S-100 (a and b) and NSE expression in human cord and placental tissue was studied by immunohistochemical analysis. Similar analysis was performed using two other brain-specific markers: glial fibrillary acidic protein and growth-associated protein B-50 (also known as GAP-43 or neuromodulin). Tissue was derived after elective cesarean section in seven women of different gestational ages after uncomplicated or complicated pregnancy. S-100 a and b and NSE immunoreactivity was found in several cell types and structures in the umbilical cord as well as in the placenta of all seven cases. Glial fibrillary acidic protein and B-50 showed no immunoreactivity. These data are of importance for interpreting findings of studies in which S-100 or NSE levels in cord blood or amniotic fluid have been related to neuronal damage in the neonate. The increased levels found may just as well be caused by leakage from placenta or umbilical cord as be caused by brain damage. We conclude that S-100 a and b and NSE are not suitable markers for neonatal brain damage. Brain-restricted proteins such as glial fibrillary acidic protein and B-50 seem more promising. **(*Pediatr Res* 51: 492–496, 2002)**

Abbreviations

GFAP, glial fibrillary acidic protein
IUGR, intrauterine growth restriction
IR, immunoreactivity
NSE, neuron-specific enolase
TBS, Tris-buffered saline

Assessment of neuronal markers in serum and cerebrospinal fluid is used in adults to detect acute and chronic brain damage (1–4). Also in perinatology, the significance of neuronal markers such as S-100 a and b and NSE in amniotic fluid, cord blood, neonatal blood, and neonatal cerebrospinal fluid has been investigated to predict brain damage in the newborn especially after perinatal asphyxia and preterm labor (5–13). Significantly higher values of these proteins were found under conditions associated with adverse neonatal outcome.

The S-100 family of proteins in the brain is thought to be neurotrophic in low concentrations but neurotoxic in high concentrations. The S-100a ($\alpha\beta$S-100) isoform is present in glial cells whereas S-100b ($\beta\beta$S-100) is present in both glial and Schwann cells (14, 15). NSE is the γ-isoenzyme of the enolases, enzymes involved in the glycolytic pathway, and is present in the cytosol of neurons (16, 17). The expression of S-100 a and b and NSE is not restricted to the nervous system (14–17). Members of the S-100 family of proteins (*e.g.* S100P, S100C, S100A11) are expressed in placental tissue (18–21). However, the expression of S-100 a and b and NSE in umbilical cord and placenta tissue has never been investigated systematically. Therefore the question arises whether the IR of S-100 a and b and NSE in (cord) blood and amniotic fluid, as observed in previous studies, indicates exclusively damage in brain tissue or may also result from protein release from placental or umbilical cord tissue.

The aim of this study was to assess the IR of S-100 a and b and NSE in placental and umbilical cord tissue. The findings may help to elucidate the question of whether S-100 and NSE are specific markers in cord blood or amniotic fluid for neonatal brain damage or whether more specific nervous tissue markers have to be used. For this purpose we investigated the expression in umbilical cord and placental tissue of S-100 a and b and NSE and compared this with the expression of GFAP and growth-associated protein B-50 (also designated GAP-43

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or neuromodulin) in uncomplicated and complicated pregnancies.

METHODS

Patients and tissue preparation. After the patients had given informed consent, we obtained umbilical cord and placenta tissues from seven women after elective cesarean section. Patient characteristics are shown in Table 1. Immediately after delivery, full-thickness biopsies were taken from the pericentral area of the placenta. Samples from the umbilical cord were taken at various locations. Tissue was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 at 4°C overnight. The material was dehydrated in ethanol. Ethanol was removed by washing in toluene overnight at 4°C. The material was embedded in paraffin.

Antibodies. Tissue was stained with antibodies reacting with S-100 a and b, the S-100 β-chain, NSE, GFAP, and B-50. Details of the antibodies and their dilutions are shown in Table 2.

Immunohistochemistry. Seven-micrometer paraffin sections were cut and mounted on slides coated with 3-aminopropyltriethoxysilane. First, sections were deparaffinized in ethanol. Then they were incubated for 30 min in TBS (0.01 M, pH 7.5 with HCl), containing H_2O_2 to inactivate the endogenous peroxidase activity. Sections were washed in TBS for 3 × 10 min. Thereafter, sections were preincubated for 30 min at 37°C to block nonspecific binding in TBS containing 3% normal goat serum when polyclonal antibody was used, and 3% normal horse serum in case of an MAb. Finally, sections were incubated with the primary antibody diluted in TBS containing 1.5% normal serum at room temperature overnight.

Next day, slices were washed in TBS for 3 × 10 min and then incubated with biotinylated secondary antibody, dilution 1:100 in TBS, for 1 h. The sections were washed in TBS and incubated in avidin biotin peroxidase reagent (Vectastain ABC-kit, elite-standard, Brunschwig, Amsterdam, The Netherlands) in TBS for 1 h at room temperature. Sections were washed in Tris HCl (0.05 M, pH 7.6) for 3 × 10 min. Staining was visualized by incubation with a diaminobenzidine solution, containing 10 mg of diaminobenzidine in 15 mL of Tris HCl with 0.024% H_2O_2. Sections were dehydrated and coverslipped in Depex (BDH Laboratory Supplies, England). Rat brain served as a positive control and sections without primary antibody as a negative control. The brains were obtained from adult, healthy, surplus Wistar rats, and the tissue was prepared similarly as placenta and cord tissue.

RESULTS

Specificity of the neural antibodies in rat brain sections. The antibodies to S-100 a and b, the S-100 β-chain, NSE, GFAP, and B-50 showed IR in rat brain. The anti-S-100 antibodies and the anti-GFAP antibody showed positive glial cells. The anti-NSE antibody and the anti-B-50 antibody showed positive neurons. All stainings were negative when no primary antibodies were added.

Table 1. *Patient characteristics*

Gestational age (weeks + days)	Fetal condition	Maternal and obstetrical pathology	Umbilical artery Doppler findings	Fetal heart rate monitoring	Birth weight in grams (percentile)*	Apgar score at 5 min	Umbilical artery pH	Neonatal pathology
29 + 6	IUGR	Preeclampsia	Abnormal	Normal	820 (<2.3)	9	7.18	Respiratory distress syndrome, bronchopulmonary dysplasia, patent ductus arteriosus, brain ultrasound abnormalities
35 + 5	IUGR	None	Abnormal	Normal	1675 (2.3)	9	7.26	Tetralogy of Fallot
31 + 0	IUGR	None	Abnormal	Abnormal	623 (<2.3)	10	7.09	Bronchopulmonary dysplasia, brain ultrasound abnormalities
39 + 4	None	Breech presentation	Normal	Normal	3100 (25–50)	9	Unknown	None
39 + 2	None	Graves disease (no medication, no antithyroid autoantibody production)	Normal	Normal	3830 (90)	10	7.31	None
36 + 4	None	Placenta previa, hypothyroidism (50 μg levothyroxine per day), heterozygous β-thalassemia	Unknown	Normal	2040 (5–10)	10	7.29	None
39 + 3	None	Adrenogenital syndrome (40 mg hydrocortisone per day)	Unknown	Normal	4120 (90)	10	Unknown	None

* Birth weight percentiles are according to the Dutch growth charts.

Table 2. *Overview of antibodies used for immunohistochemical staining*

Antibody*	Concentration	Supplier
Anti-S-100 a and b (p)	1:800	DAKO, Glostrup, Denmark
Anti-S-100 β chain (p)	1:400	Santa Cruz Biotechnology, Inc, Santa Cruz, CA, U.S.A.
Anti-NSE (m)	1:1600	Sanbio, Am Uden, the Netherlands
Anti-GFAP (p)	1:1600	Roche Molecular Biochemicals, Mannheim, Germany
Anti-GFAP (m)	1:1600	Roche Molecular Biochemicals
Anti-B-50 (m)	1:200,000	Characterized by Mercken *et al.* 22
Anti-B-50 (p)	1:100,000	Characterized by Aarts *et al.* 23

* m, monoclonal; p, polyclonal.

S-100 staining. Immunohistochemical analysis of both the anti-S-100 a and b and the anti-S-100 β-chain antibodies showed IR in the umbilical cord, owing to positive staining of the smooth muscle cells of the vascular wall, myofibroblasts in Wharton's jelly, amnion epithelium, macrophages, and monocytes (Fig. 1, *A* and *B*). In the placenta the anti-S100 antibodies showed IR of the syncytiotrophoblast, myofibroblasts, smooth muscle cells of the vascular wall, and macrophages (Fig. 2*A*).

NSE staining. In the umbilical cord the NSE antibody showed IR of the smooth muscle cells of the vascular wall, endothelium, myofibroblasts in Wharton's jelly, amnion epithelium, macrophages, and monocytes (Fig. 1*C*). In the placenta the NSE antibody stained the syncytiotrophoblast, myofibroblasts, macrophages, smooth muscle cells of the vascular wall, and the endothelium (Fig. 2*B*).

GFAP and B-50 staining. The GFAP and the B-50 antibodies showed no IR in umbilical cord or placenta. A summary of the results of the immunohistochemical staining is given in Table 3. The results were the same in tissues obtained from all pregnancies.

DISCUSSION

We demonstrated IR for the S-100 protein (a and b and the S-100 β-chain) and NSE in several cell types and structures of umbilical cord and placental tissue. In contrast to these find-

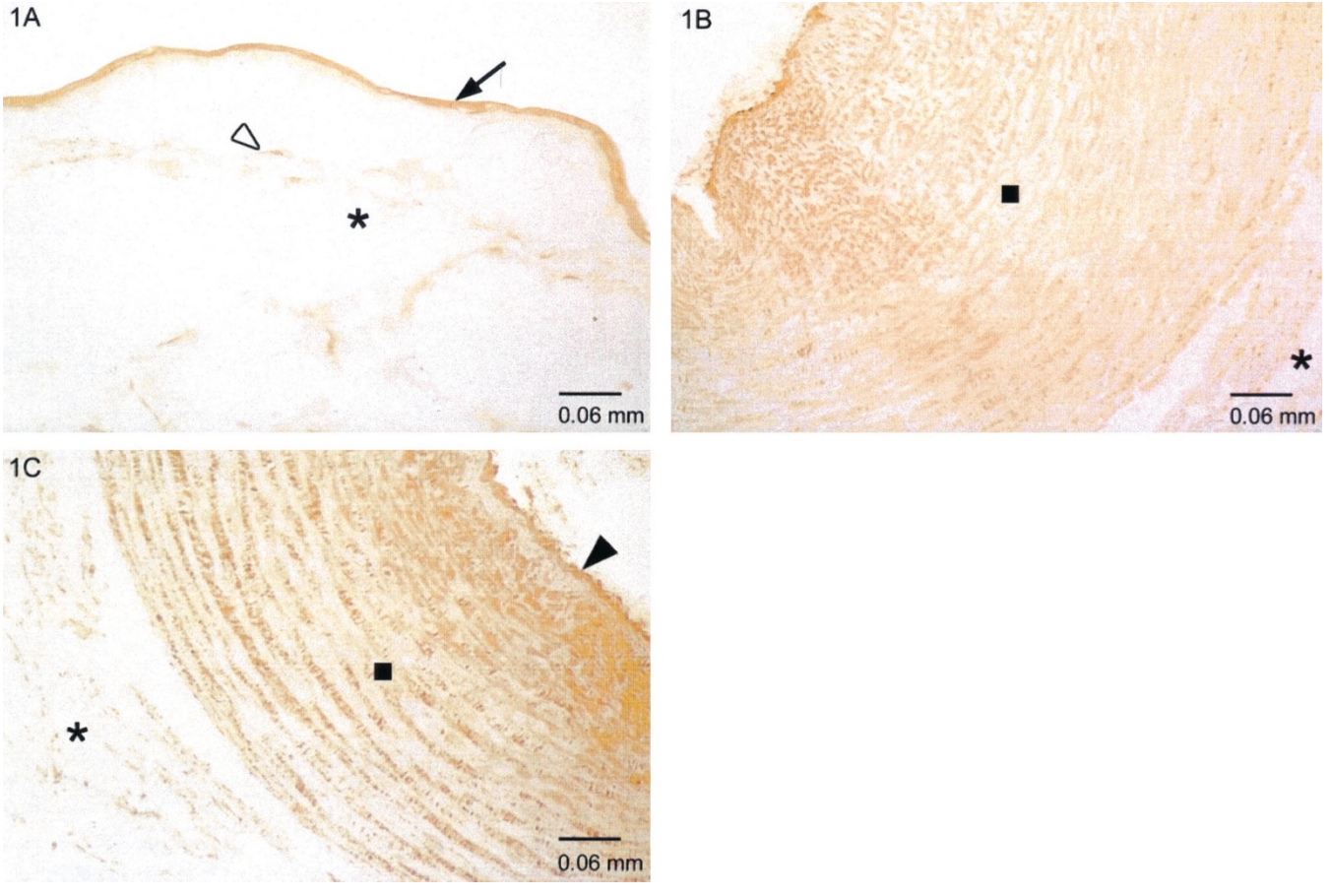

Figure 1. Expression of markers in umbilical cord tissue. Expression of the S-100 a and b antibody in myofibroblasts in Wharton's jelly and amnion epithelium (*A*) and in smooth muscle cells of the vascular wall (*B*). The S-100 β-chain antibody showed the same IR. *C*, NSE IR in the smooth muscle cells of the vascular wall and endothelium of an artery. The myofibroblasts in the Wharton's jelly also showed NSE IR. *, Wharton's jelly; ▷, myofibroblast; →, amnion epithelium; ►, endothelium; ■, vascular wall.

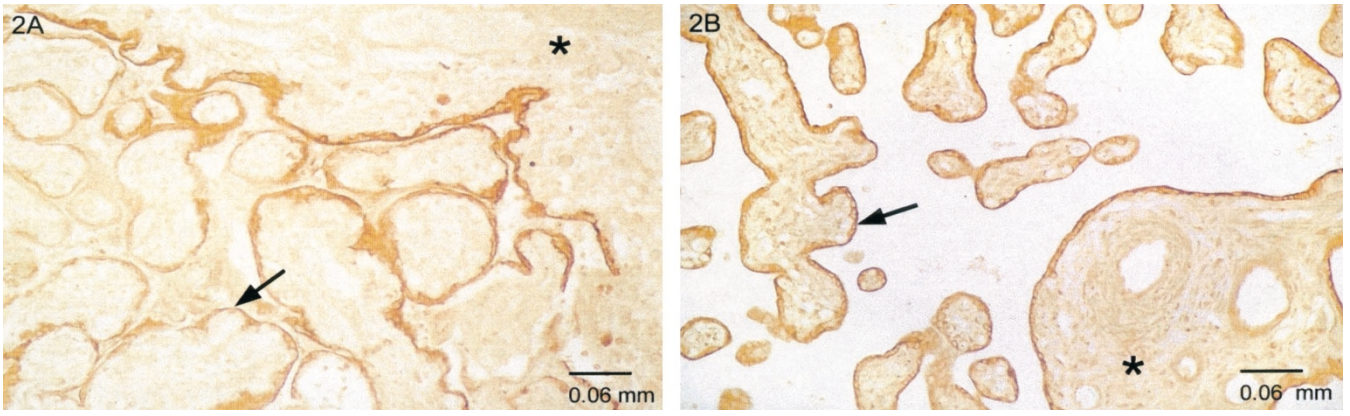

Figure 2. Expression of neuronal markers in placenta tissue. *A*, S-100 expression in syncytiotrophoblast, myofibroblasts in stroma, and macrophages. *B*, NSE IR of syncytiotrophoblast and myofibroblasts in stroma. *, stem villi; →, trophoblast.

Table 3. *Overview of results of staining with S-100, NSE, GFAP, and B-50*

	Umbilical cord staining		Placenta staining	
Antibody*	+/−	Cells and structures involved	+/−	Cells and structures involved
S-100 a and b	+	smooth muscle cells of the vascular wall, myofibroblasts, amnion epithelium, macrophages, and monocytes	+	syncytiotrophoblast, myofibroblasts, smooth muscle cells of the vascular wall, and macrophages
S-100 β chain	+	smooth muscle cells of the vascular wall, myofibroblasts, amnion epithelium, macrophages, and monocytes	+	syncytiotrophoblast, myofibroblasts, smooth muscle cells of the vascular wall, and macrophages
NSE	+	smooth muscle cells of the vascular wall, myofibroblasts, amnion epithelium, macrophages, monocytes, and endothelium	+	syncytiotrophoblast, myofibroblasts, smooth muscle cells of the vascular wall, macrophages, and endothelium
GFAP (p)/GFAP (m)	−		−	
B-50 (m)/B-50 (p)	−		−	

* m, monoclonal; p, polyclonal.

ings, no IR of the glial-specific marker GFAP or the neuronal-specific marker B-50 could be found in any of the investigated tissues. The results were consistently found in the tissues obtained from the three IUGR and the four uncomplicated pregnancies.

Levels of NSE and S-100 have been determined quantitatively in cord blood after normal pregnancies to obtain reference values (24, 25) or to relate levels to gestational age in term and preterm pregnancies (26). To have an indication of brain damage after adverse perinatal conditions, the presence of neuronal-specific proteins in blood and amniotic fluid has been investigated. In amniotic fluid, increased NSE levels have been found in pregnancies with subsequent adverse neonatal neurologic outcome (5, 11). Di Iorio *et al.* (12) determined S-100 in cord blood from IUGR fetuses. They found lower S-100 values when nitric oxide was given to the mother to increase uteroplacental circulation. The authors suggested that IUGR fetuses may have neuronal cell injury.

The presence of S-100 a and b and the S-100 β-chain and NSE IR in several structures and cells of cord and placenta tissue questions the validity of these proteins as neuronal markers in cord blood. Increased leakage from a damaged placenta caused by villitis or infarction may explain elevated (cord) blood and amniotic fluid S-100 and NSE levels, and a relation with adverse neurologic outcome may be indirect rather than direct. The presence of S-100 a and b and NSE in cord and placental tissue has to our knowledge not been studied before. However, not only the presence of the proteins in placental and cord tissue has to be taken into consideration. Brown *et al.* (2) found relatively high levels of NSE in erythrocytes. They concluded that NSE is not a useful marker in blood unless hemolysis of erythrocytes is adequately assessed. Elimian *et al.* (11) suggested the development of an assay to distinguish between NSE released from erythrocytes containing one γ subunit and NSE measured after neuronal damage, which contains the γγ isoenzyme. Moreover, S-100 has a short biologic half-life, 25–40 min according to a recent publication and approximately 2 h according to other investigators (27, 28). This makes assessment of brain damage by means of S-100 determination only valuable after acute situations or in conditions with a continuous protein release from the brain.

Because of these arguments and the results of our findings, our opinion is that there may be a relation between damage in the nervous system and elevated NSE and S-100 in, for example, (cord) blood, but this relation might not be causal.

The direct assessment of neurologically adverse effects in the newborn by means of markers in peripheral blood is of great clinical significance. First, knowledge of neurologic damage could be helpful after a serious compromising event to decide whether to start treatment and to determine which treatment should be started. Next, serial measurements of

brain-specific proteins can be used to monitor effects of pharmacologic interventions to protect the brain. Third, determination of brain-specific markers may be valuable in the evaluation of possibly adverse or beneficial effects of new interventions. According to our results, S-100 a and b and NSE seem to be not specific enough to serve as markers of brain damage in cord blood or amniotic fluid. Serial measurements of NSE and S-100 in one neonate, which means measurement in cord blood and sequential measurements after birth in neonatal blood, might help to distinguish between protein released from placental and umbilical cord tissue or from tissue of neonatal origin. However, even when one could distinguish between placental or cord origin and neonatal origin, the question remains whether S-100 or NSE from the neonate is coming from nervous tissue (14–17, 29).

We also studied the expression of GFAP and B-50. GFAP is a filament protein expressed in the cytoplasm of astrocytes (7). B-50 is a growth-associated presynaptic protein, expressed in neurons (30). Although B-50 is mainly attached to the membrane in the adult situation, during development approximately 50% of the protein is present in the cytoplasm. GFAP and B-50 seem promising as markers for neuronal damage in the neonate inasmuch as no IR of these proteins was found in placenta or umbilical cord.

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