

# Astrocytic-Inducible Nitric Oxide Synthase in the Ischemic Developing Human Brain

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**ABSTRACT:** Variability in the expression of apoptotic and inflammatory mediators with time after an ischemic insult and their role in the expansion of cerebral infarcts are still controversial. This study examines DNA degradation and the expression of activated caspase-3 and iNOS, inducible nitric oxide (iNOS) in the human developing brain. Autopsy specimens from children with a neuropathologic diagnosis of focal ischemic infarct were included in the study. The specimens were classified based on the clinical history as acute (<24 h,  $n = 5$ ), subacute (24–72 h,  $n = 8$ ), or old (>72 h,  $n = 6$ ) infarcts. Immunohistochemical staining for caspase-3, iNOS and TUNEL were then performed on all infarcts alongside age-matched controls. TUNEL staining was detected in regions of all infarcts. Expression of iNOS was significantly higher than that of caspase-3 in the penumbra of subacute infarcts ( $p = 0.02$ ). Glial fibrillary acidic protein and iNOS staining co-localized in the penumbra of acute and subacute infarcts. These results suggest that cell death continues to occur for more than 3 d post ischemic insult. Cell death in the penumbra of subacute infarcts is partially caspase-3 independent and may be attributed to nitric oxide. Astrocytes are a source of iNOS and may play a role in the evolution of pediatric brain injury days after the initial insult. (*Pediatr Res* 60: 687–692, 2006)

Cerebrovascular disorders are among the top 10 causes of death in children, with rates being highest in the first year of life. Population-based studies have identified incidence rates of pediatric ischemic stroke of 3.3–13/100,000 (1–3). The incidence rate of neonatal stroke is approximately 1/4000 live births per year (4,5). Incidence and prevalence of stroke in children is likely to increase because of increased awareness and improvement in radiographic diagnosis. Although several studies have been published on the pathophysiology of infarcts in the adult brain, very little is known about the pathophysiology of pediatric infarcts.

Studies using animal models of neonatal stroke have shown that activated caspase-3 (6–8) and nitric oxide (9–11) are mediators of ischemic injury in the developing brain. Several studies (12–15) have shown that necrotic cell death predominated in the ischemic core whereas apoptosis occurred in the penumbra. A more recent study showed that neuronal caspase-3 was expressed in both the ischemic core and penumbra after transient focal ischemia with a more rapid in-

crease in the penumbra (16). Nitric oxide synthase (NOS) activity, on the other hand, was shown to be higher in the core compared with the penumbra (9). Furthermore, mice lacking iNOS are less vulnerable to ischemic injury compared with wild type (10), suggesting iNOS blockade may serve to limit the extent of ischemic damage.

Glial cells are thought to be more resistant to injury in the CNS. However, astrocytic swelling is an early response to ischemic injury. Following focal ischemia, astrocytes will increase uptake of glutamate,  $K^+$  ions, and lactate in an attempt to reduce the accumulation of these molecules in the extracellular space. Consequently, astrocytes will swell leading to cerebral edema that will exacerbate the ischemic injury and cause death (17). In addition to trying to protect the homeostasis of the extracellular milieu, astrocytes maintain their ability to communicate with each other and with distant neurons through gap junctions that remain open during an ischemic injury (18). Consequently, proapoptotic substances such as iNOS, shown to be expressed in rat glial cells following ischemia (11,19,20), can diffuse to neighboring cells via this network and cause further damage.

The variability in the expression of cell death mediators with time after an ischemic insult and their role in the expansion of cerebral infarcts are still controversial, however (6–8). Moreover, attempts to extrapolate from animal models have limitations when it comes to clinical applications. The objective of this study was to examine the regional distribution and the time course of caspase-3- and iNOS-mediated injury following ischemia in the human developing brain using clinically defined acute, subacute and old infarcts. A better understanding of the role of these proteins in human pediatric ischemic infarcts may point toward useful therapeutic targets.

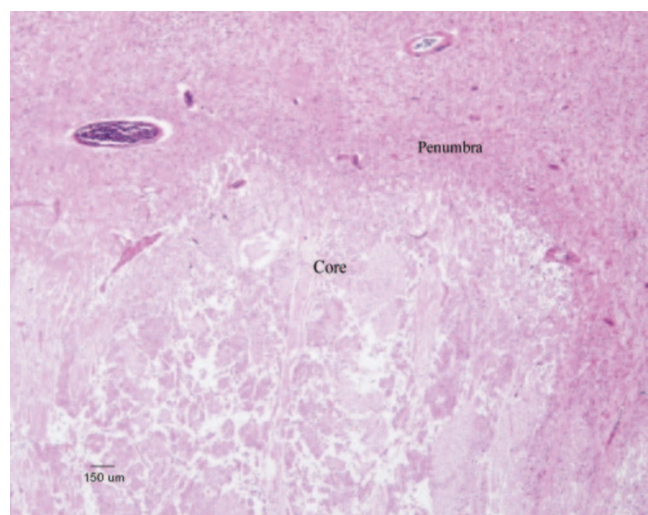
## PATIENTS AND METHODS

**Patient population.** The database in the Department of Pathology at the Hospital for Sick Children was searched for autopsy specimens from children registered from January 1990 to July 2004 with a neuropathologic diagnosis of cerebral ischemic infarct. Specimens were included in the study if 1) patient was born at term; 2) patient age <18 y; and 3) cerebral infarct had well-defined borders. Patients with global ischemia were excluded from the study. After reviewing the charts, infarcts were classified based on the clinical

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**Abbreviations:** GFAP, glial fibrillary acidic protein; iNOS, inducible nitric oxide



**Figure 1.** Hematoxylin and eosin (H&E) staining of subacute infarct in the pons of a 3-wk-old female who died of neonatal meningitis. Core of the infarct was defined by loss of normal H&E staining pattern of brain parenchyma with specific cellular changes depending on the age of the infarct. Normal brain parenchyma directly adjacent to the ischemic core (arrow) is the ischemic margin or “penumbra.”

history as acute if the time between clinical neurologic compromise and autopsy was up to 24 h, as subacute if that time was 24–72 h, and old if more than 72 h. Age-matched brain tissue sections with normal histology were used as controls. The study was approved by the Research Ethics Board at our institution.

**Neuropathological examination.** Paraffin-embedded sections of postmortem pediatric human brains stained with hematoxylin and eosin (H&E) were reviewed by the neuropathologist (C.H.) to confirm the diagnosis of focal ischemic infarct. The area of the infarct was defined by loss of normal H&E staining pattern of brain parenchyma with specific cellular changes depending on the age of the infarct. Acute infarcts showed neuronal eosinophilia with nuclear pyknosis and vacuolation of surrounding neuropil. In addition, neutrophil and early macrophage infiltration was seen in the subacute infarcts. Vascular proliferation, cystic cavity formation, abundant foamy histocytes, and reactive astrocytosis were characteristics of old infarcts. Brain parenchyma directly adjacent to the ischemic “core” was defined as the ischemic margin or “penumbra” (Fig. 1). None of the age-matched control sections showed any pathologic changes.

**Tissue preparation.** Five micrometers of formalin fixed, paraffin embedded tissue section were mounted on positive-charged microscope slides. Tissue sections were then baked overnight at 60°C, de-waxed in xylene, and hydrated to distilled water through decreasing concentrations of alcohol.

**TUNEL assay.** DNA damaged cells were detected using the *in situ* end labeling assay for TUNEL (terminal deoxynucleotidyl transferase-mediated DNA nick-end labeling) adapted to an automated immunohistochemical hybridization technique (Discovery Ventana Medical Systems, Inc., Tucson, AZ). Before staining, the sections were blocked for endogenous peroxidase and a biotin block, with subsequent online digestion with protease I (Ventana Medical Systems) for 12 min. The assay uses recombinant terminal deoxynucleotidyl transferase (Tdt) (Invitrogen, Carlsbad, CA) for adding homopolymer tails to the 3' ends of cleaved DNA, characteristic in cells undergoing programmed cell death. Biotin 16-dUTP (Roche Diagnostics, Basel, Switzerland) was the label used for this reaction. Colorimetric visualization using avidin-horse radish peroxidase and 3,3'-diaminobenzidine (DAB) detection method was performed. The counterstain was hematoxylin.

**Activated caspase-3.** Antibody against cleaved caspase-3 (Cell Signaling Technology, Beverly, MA) was pretreated with a heat-induced epitope retrieval technique using citrate buffer at pH 6.0, and used at a dilution of 1/25. Immunohistochemical procedures were performed on the Ventana NEXES auto-immunostainer (Ventana Medical Systems). Immunodetection was performed using an ABC (avidin/biotin complex) system, employing the Ventana *i*-View DAB (3,3'-diaminobenzidine) Detection Kit. All tissue sections were treated for endogenous peroxidase and biotin. The counterstain of preference was hematoxylin, for nuclear detail.

**iNOS staining.** The antibody against iNOS (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) required protease I (Ventana Medical Systems) pretreatment for 16 min as part of the automated staining protocol. The antibody

was then used at a dilution of 1/40. The use of this antibody to detect iNOS is well documented in the literature (45,46).

**TUNEL and neurofilament.** Antibody for neurofilament is a well-established marker for detecting neurons. Double staining with neurofilament and TUNEL was done to identify the type of the dying cells. TUNEL staining was carried out as described above. Following that, the immunohistochemical procedure for neurofilament (Sanbio, Am Uden, the Netherlands) was performed. Sections were retreated with endogenous peroxidase and biotin before incubation with neurofilament at 1/50 dilution for 60 min at room temperature. Immunodetection was performed employing the Elite ABC detection system (Vector Laboratories, Burlingame, CA), as described by the manufacturer, in conjunction with the Vector VIP substrate kit (Vector Laboratories), as the chromogen, producing a purple reaction product. The counterstain of preference was hematoxylin, for blue nuclear detail.

**GFAP and iNOS.** Antibodies to GFAP are routinely used to identify glial cells particularly astrocytes. The morphology of iNOS-positive cells suggests that these cells are astrocytes. To confirm the identity of iNOS-positive cells by immunocytochemistry, we co-stained them with antibodies against iNOS and GFAP. Sections were digested with 0.5% pepsin at 37°C for 30 min, and blocked with biotin and goat normal serum before the application of the primary antibody. The incubation of anti-human GFAP (DAKO, Carpinteria, CA) at 1:1000 dilution was carried out at room temperature for 1 h, followed by anti-rabbit IgG secondary antibody and fluorescent-streptavidin (Vector Laboratories, Inc.). Anti-iNOS antibody (Santa Cruz Biotechnology) at 1:10 dilution was added to the slides and incubated at 4°C overnight. Then, the anti-mouse IgG secondary antibody and TRITC-streptavidin (Vector Laboratories Inc.) was applied to the slides and incubated at room temperature for 30 min. The slides were washed with 1× PBS and cover-slipped.

**Cell counting.** Cell counting was performed independently by two investigators blinded to the clinical classification of infarcts (R.A. and C.H.) in the core and margin of each infarct. Averages of four to five visual fields were counted for each region at 40× magnification. The same areas were counted in the age-matched control brains for each immunostain. The numbers of positive cells were reported as mean ± SD.

**Statistical Analysis.** The means ± SD of positive cells counted for different stains in the core and margin of each infarct were compared among acute, subacute and old infarcts. Statistically significant differences were analyzed using one-way analysis of variance.

## RESULTS

A total of 19 patients registered in the database of the Department of Pathology in our institution from January 1990 to July 2004 with a neuropathological diagnosis of cerebral ischemic infarct met the inclusion criteria of our study. Of these 19 patients, 5 had acute infarcts, 8 had subacute infarcts, and 6 had old infarcts. None of these patients died of their ischemic infarcts. The clinical data on patients and controls ( $n = 5$ ) are shown in Table 1.

**DNA damage.** DNA damage, as detected in tissue sections using TUNEL immunostaining was significantly increased compared with control in the core and penumbra of all infarcts at all ages (representative sections are shown in Fig. 2). There was no statistically significant difference in the number of TUNEL-positive cells between the core and penumbra in all infarct ages (Table 2). Only rare TUNEL-positive cells were seen in the control sections ( $26 \pm 12$ ) and in the nonaffected areas outside the penumbra. TUNEL and neurofilament staining were detected in the same cells of all infarcts (Fig. 3), confirming the damaged cells to be neurons.

**Caspase-3 activity.** There were large numbers of caspase-3-positive cells in the core and penumbra of acute, subacute, and old infarcts (Fig. 4). However, the number of caspase-3-positive cells significantly decreased in the penumbra of subacute compared with acute infarcts (Table 2). The number of caspase-3-positive cells was significantly less than the number of TUNEL-positive cells (Table 2) in the core ( $p < 0.0001$ ) and penumbra ( $p = 0.01$ ) of subacute infarcts. No significant

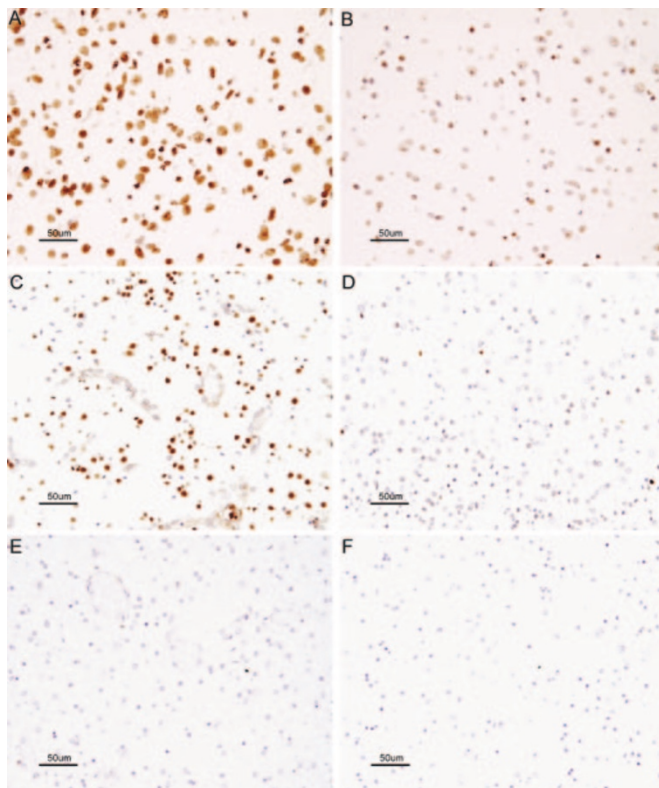
**Table 1.** Clinical data for patients and controls included in the study

Case	Sex/age	Infarct territory	Cause of death	Infarct type
1	F/1.5 wk	R hippocampus	Perinatal asphyxia	Acute
2	M/6 y	R frontal cortex	CHD/Williams syndrome	Acute
3	M/4 y	Pons	ALL/Extensive pulmonary hemorrhage	Acute
4	M/10 mo	L MCA	Down's syndrome/CHD	Acute
5	F/9 y	Midbrain	Trauma	Acute
6	M/3 mo	L thalamus	CHD/multiorgan failure	Subacute
7	F/3 wk	Pons	Sepsis	Subacute
8	F/3 y	L thalamus	CHD/multiorgan failure	Subacute
9	M/1 wk	L temporal cortex	CHD	Subacute
10	F/13 y	R basal ganglia	Dilated cardiomyopathy/ARDS	Subacute
11	M/1 y	Pons	Dilated cardiomyopathy	Subacute
12	F/5.5 mo	Medulla	Cardiac arrest of unknown etiology	Subacute
13	M/1.5 wk	L occipital cortex	Diffused alveolar damage	Subacute
14	M/6 mo	L calcarine cortex	Transposition of great vessels	Old
15	M/16 y	L frontal cortex	CHD/post cardiac transplant	Old
16	F/1 y	L calcarine cortex	Down's syndrome/CHD	Old
17	F/11 y	R frontotemporal cortex	CP/peritonitis following G-tube insertion	Old
18	F/5 y	R frontal cortex	Autoimmune hemolytic anemia	Old
19	M/11 y	Midbrain	High cervical cord injury	Old
Controls		Normal brain sections		
1	F/9 y	R frontal cortex/midbrain	Anaplastic large cell lymphoma	
2	M/2 mo	L thalamus	Sudden death	
3	F/1 y	L calcarine cortex	Asphyxia	
4	F/13 y	R basal ganglia	Multiorgan failure post bone marrow transplant	
5	F/3 wk	Pons/L hippocampus	Sudden Death	

R, right; L, left; ALL, acute lymphocytic leukemia; CHD, congenital heart disease; ARDS, acute respiratory distress syndrome; CP, cerebral palsy.

difference between the two stains was found in the acute and old infarcts. Rare Caspase-3-positive cells were seen in the control sections ( $3 \pm 6$ ) and in the nonaffected areas outside the penumbra.

**iNOS staining.** There were large numbers of iNOS-positive cells in the core and penumbra of all infarcts at all ages. Very rare iNOS-positive cells were observed in controls ( $2 \pm 2$ ) and in the nonaffected areas outside the penumbra. There was a significant decrease in the number of iNOS-positive cells (Fig. 5) in the core ( $p < 0.05$ ) but not the penumbra of subacute compared with acute infarcts. The number of iNOS-positive cells was significantly higher ( $p = 0.02$ ) in the penumbra versus the core of subacute infarcts (Table 2). Comparing caspase-3 to iNOS staining, the number of caspase-3-positive cells was significantly higher in the core ( $p = 0.03$ ), whereas iNOS-positive cells were higher in the penumbra ( $p = 0.04$ ) of subacute infarcts. TUNEL staining, on the other hand, was



**Figure 2.** TUNEL staining in core (A,C,E) and penumbra (B,D,F) of acute (A–B), subacute (C,D), and old (E,F) infarcts in the hippocampus, left temporal lobe, and right frontal cortex, respectively.

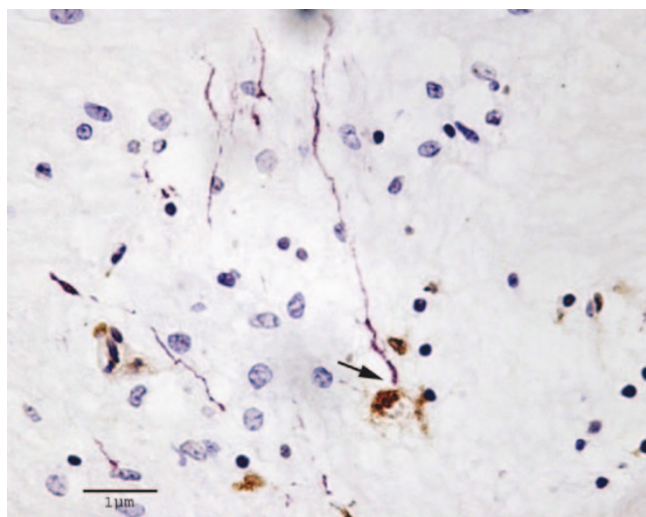
**Table 2.** Mean number of immuno-positive cells in acute, subacute, and old infarcts

Stain	Acute infarcts	Subacute infarcts	Old infarcts
TUNEL*	67 ± 34	77 ± 18	53 ± 33
TUNEL**	65 ± 35	52 ± 26	59 ± 34
Caspase-3*	52 ± 16	30 ± 7	30 ± 26
Caspase-3**	41 ± 23	15 ± 5	25 ± 10
iNOS*	42 ± 4	22 ± 5	33 ± 21
iNOS**	46 ± 18	37 ± 14	35 ± 18

Data reported as mean + SD of 4–5 high-power (40×) fields. For statistical data, see text.

\* Core; \*\* penumbra.

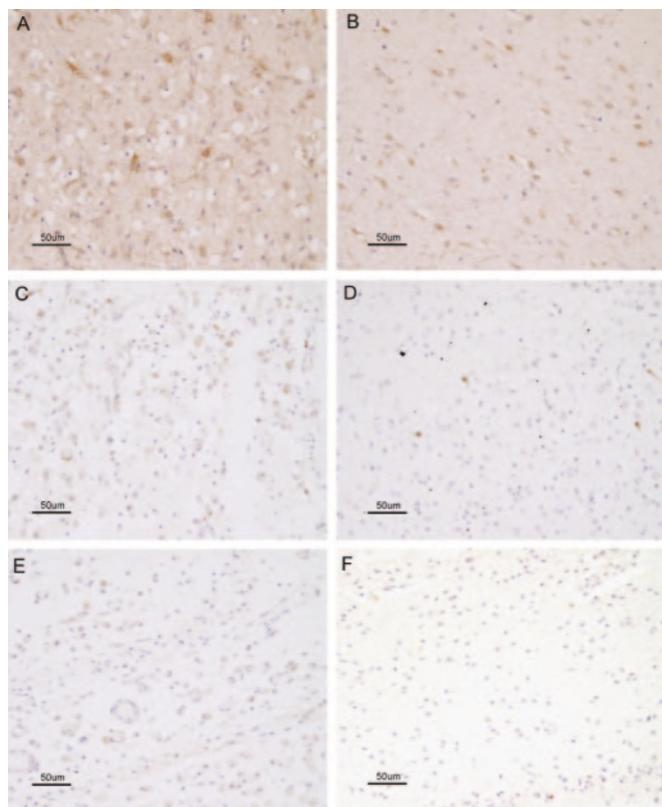




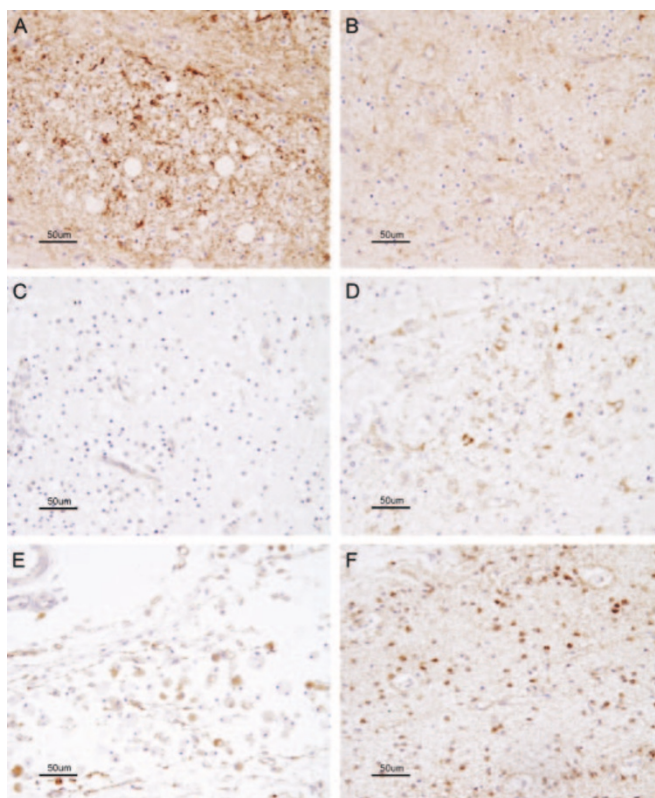
**Figure 3.** TUNEL-positive cells are neurons. TUNEL staining and neurofilament immunostaining were detected in the same cell (arrow).

significantly higher ( $p < 0.0001$ ) when compared with iNOS staining in the core but not in the penumbra ( $p = 0.2$ ) of subacute infarcts. These differences were not observed in the core and penumbra of acute and old infarcts (Table 2).

**GFAP and iNOS staining.** Many iNOS-positive cells were also positive for GFAP staining in the penumbra of acute and subacute infarcts (Fig. 6). None of the control sections stained



**Figure 4.** Activated caspase-3 detected by immunohistochemistry in core (A,C,E) and penumbra (B,D,F) of acute (A,B), subacute (C,D), and old (E,F) infarcts in the hippocampus, left temporal lobe, and left calcarine cortex, respectively.



**Figure 5.** iNOS immunohistochemical staining in core (A,C,E) and penumbra (B,D,F) of acute (A,B), subacute (C,D), and old (E,F) infarcts in the pons, left temporal lobe, and left calcarine cortex, respectively.

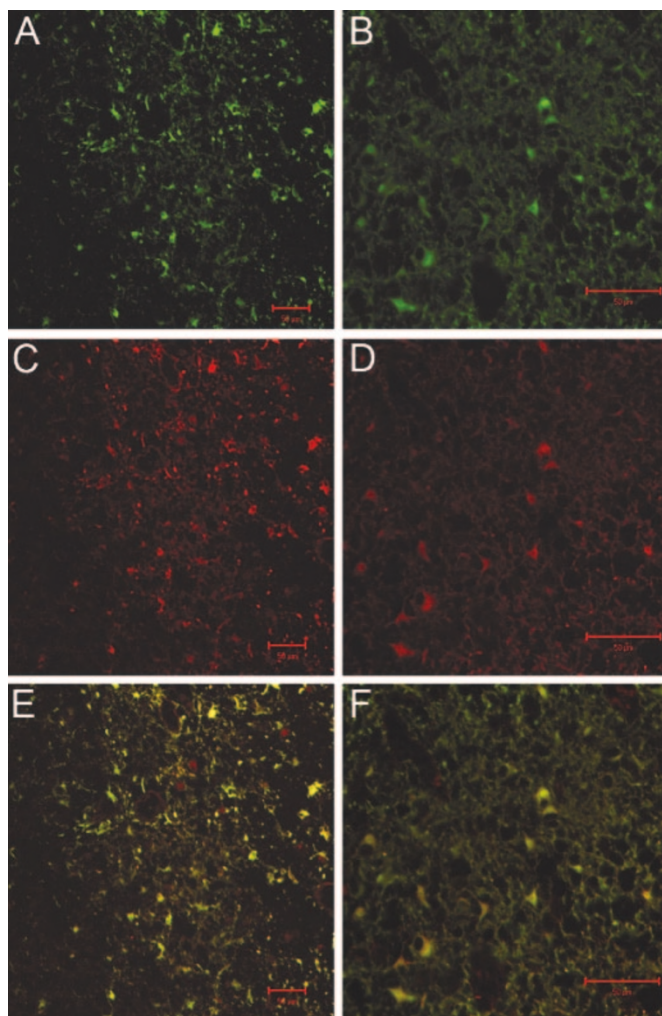
with GFAP had iNOS-positive cells. These results indicate that the iNOS is being produced by astrocytes in the penumbra up to 72 h post ischemic infarct.

## DISCUSSION

This is one of very few studies to look at the mechanism of injury in the human developing brain after an ischemic insult. We report four important findings: 1) cell death continues to occur in neurons in the core and penumbra of stroke for more than 72 h; 2) prolonged activation of caspase-3 in core and penumbra beyond 72 h; 3) iNOS prolonged expression in the core and penumbra of the infarct for more than 72 h; and 4) iNOS is expressed in astrocytes following ischemic injury.

TUNEL staining remained high in acute, subacute, and old infarcts, showing that cell death continued to occur in the core and penumbra of stroke for more than 72 h. This is consistent with what Pulera *et al.* (7) and Zhu *et al.* (8) have shown where TUNEL positivity was detected 72 h and 48 h, respectively, following ischemic insult in neonatal rats. However, in adult rat brain the peak of TUNEL immunostaining was between 12 and 24 h following focal ischemia (21).

We found that activated caspase-3 continued to be detected for more than 72 h after the ischemic insult. Our findings are supported by the work of others. Pulera *et al.* (7) reported the detection of apoptosis at 1, 3, and 4 d after ischemic insult in a neonatal stroke model using two antibodies specific for activated caspase-3. More recently, caspase-3 reactivity was reported at 24 and 72 h (7,8) after ischemic



**Figure 6.** Localization of iNOS in astrocytes. Penumbra of acute (A,C,E) and subacute (B,D,F) were stained with GFAP antibody (A,B) and iNOS MAB (C,D) and were visualized with FITC-streptavidin (green) and TRITC-streptavidin (red), respectively. GFAP-positive cells were co-stained with iNOS as shown by merging the two images in E and F (yellow).

injury in a neonatal animal stroke model and remained high in some regions of the brain up to 7 d after the insult (8). In adult mouse brain, activated caspase-3 was detected 12–24 h after reperfusion (22). These data, taken together, suggest that cell death in the developing brain continues for longer periods of time compared with mature brain after an ischemic insult.

In the subacute infarcts, the number of cells staining with caspase-3 is less than that staining with TUNEL suggesting both caspase-3-dependent and -independent cell death. Caspase-3 and TUNEL reactivity are not significantly different in the acute and old infarcts, suggesting that cell death is primarily caspase-3 dependent at these stages. In contrast, Zhu *et al.* (8) reported that caspase-3 positivity exceeded TUNEL positivity at 3 h post ischemia, but the two stains completely co-localized at 24 h in a neonatal stroke model.

The above results taken together suggest that cell death in the developing brain is ongoing for at least 72 h following an ischemic insult. A similar prolonged cell death has not been shown in adult brain yet. Caspase-3-dependent cell death may

predominate acutely (first 24 h) but in subacute infarcts caspase-3-independent pathways start playing a role in mediating cell death.

A potential weakness of this study, necessitated by the scarcity of human tissue, is the broad age range of included patients (1 wk to 16 y of age) as well as the differences in infarct location. To address this we examined our data to look for potential differences in immuno-positive cell counts related to patient age (<6 mo *versus* >6 mo) or infarct location (infra- *versus* supratentorial). There were no significant differences in our data in the number of TUNEL-positive or caspase-3- or iNOS-immuno-positive cells in the acute and subacute infarcts based on patient age or infarct location (data not shown). Nevertheless, as our study was not designed to address this question, we cannot exclude the possibility that the role of caspase-3-dependent and -independent pathways may vary as the brain matures within this pediatric population.

iNOS is expressed in the core and penumbra of the infarcted developing human brain for more than 72 h following the ischemic insult with a significant drop in iNOS expression after 24 h in the core but not the penumbra of subacute infarcts. Expression of iNOS in the penumbra of subacute infarcts is significantly higher than the expression of activated caspase-3, whereas the reverse is true in the infarct core. These results suggest that iNOS-mediate cell death in the penumbra in part, independently of caspase-3, while caspase-3-mediated cell death predominates in the core of subacute infarcts in the developing brain.

The fact that iNOS expression was still detected in the penumbra of infarcts older than 72 h is in agreement with studies showing that selective iNOS inhibition was neuroprotective 72 h post ischemia in the adult (23,24) and neonatal (25) rat hypoxic-ischemic model. In contrast, a decrease in NOS activity was reported by 24 h post ischemia in the rat pup (9). This reported early decrease in NOS activity may be attributed to neuronal NOS (nNOS) activity (26) and not iNOS.

Co-localization of iNOS and GFAP staining suggests that iNOS is expressed in astrocytes following ischemic injury in the developing brain. These results are consistent with previous reports showing iNOS expression by reactive astrocytes in adult rat (19) and neonatal rat cultured glial cells (20) following ischemia. However, in autopsy adult brains with neuropathological evidence of acute cerebral infarction, iNOS immunoreactivity was observed in neutrophils infiltrating the infarcted brain and in blood vessels within the infarcted territory (27). Expression of iNOS was shown predominately in neutrophils, and smooth muscle cells but not astrocytes in immature rats (28) and adult human brain (29) following traumatic brain injury. Our study provides evidence that astrocytes are a source of iNOS expression in the human developing brain following ischemia.

The physiologic role of astrocytic iNOS production following ischemia is not clear yet. Astrocytes are a major source of NO (30) but they also protect against oxidant damage (31–33). They contain the highest concentrations of antioxidants in the brain and provide neurons with extracellular precursors for neuronal glutathione (GSH) synthesis (33,34). Therefore, one



possible mechanism of iNOS-induced ischemic injury is that increased expression of iNOS and subsequently NO may deplete cellular glutathione resulting in potentiation of ischemic injury as shown in adult (35–37) and neonatal (38) rat brain. Another possible mechanism of NO-induced ischemic injury is by disrupting astrocytic networks. It has been shown that NO uncouples gap junctions (39), which play an important role in neuronal survival following an ischemic injury (17,40). Elevation of neuronal  $Ca^{2+}$  induced by oxidative injury was larger with uncoupling treatment (41), suggesting that astrocytic gap junctions were also involved in neuronal intracellular  $Ca^{2+}$  homeostasis. However, nonselective nitric oxide synthase inhibitor did not alter the enhancement of oxidative neuronal injury by uncoupling of gap junction in mixed cultures of neurons and astrocytes (42). The deleterious effects of inflammatory iNOS expression to the ischemic brain are well documented in the literature (for reviews, see Refs. 26,43,44). However, the role of astrocytic iNOS expression in the context of ischemic injury is not clear yet. Further work is required to elucidate astrocyte-neuron communication following ischemia in the developing brain.

In summary, cell death continues to occur in the core and penumbra of stroke for more than 72 h in the human developing brain. This ongoing cell death is mediated by both caspase-dependent and caspase-independent mechanisms. iNOS-mediated cell death is shown to predominate in the penumbra of subacute infarcts, implicating it as a possible therapeutic target with a good therapeutic window.

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