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RESEARCH ARTICLE

Expression of Cell Death-Associated Proteins in Neuronal Apoptosis Associated with Pontosubicular Neuron Necrosis

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Expression of apoptosis-associated proteins p53, bcl-2, bax, and caspase-3/CPP32, activation of caspase-3, and modification of proteins via poly(ADP-ribosyl)ation was studied in pontosubicular neuron necrosis (PSN), a form of perinatal brain damage revealing the morphological hallmarks of neuronal apoptosis. Immunoreactivity for p53 was completely absent. The majority of cells stained with the bax and procaspase-3 antibodies did not show morphological signs of apoptosis. In contrast, an antibody against activated caspase-3 almost exclusively stained cells with apoptotic morphology. Poly(ADP-ribosyl)ated proteins were only rarely detected in cells with apoptotic morphology. The expression patterns of bax, procaspase-3, bcl-2, and p53 in PSN were similar to that found in age-matched control brains. However, activated caspase-3 and poly-ADP-ribosylated proteins were exclusively found in apoptotic cells. These data indicate that detection of active caspase-3 is a reliable marker for apoptosis in formalin-fixed human tissue, and that neuronal apoptosis in pontosubicular neuron necrosis is accompanied by a pronounced activation of caspase-3.

Introduction

Pontosubicular neuron necrosis (PSN) is a special form of perinatal hypoxic brain damage characterized by nerve cell apoptosis mainly in the subicular segment of the hippocampus and in the ventral pontine nuclei (5, 13). The vulnerable period for manifestation of the typical lesion extends form the thirtieth gestational week to the second postnatal month (13). The pathogenesis of PSN is still unknown. A stereotypic neuronal response to hypoxia, acute ischemia, hypoglycemia, hypocapnia, or hyperoxygenation in a critical developmental frame is suspected (12, 13, 24, 39, 54). An association with intraventricular hemorrhage has also been reported (51).

The lesions were originally described as acute neuronal necrosis showing karyorrhexis with loss of definition of the nuclear membrane and disintegration of the nucleus into basophilic granules or into irregular lumps of basophilic material (13). However, the apoptotic nature of neuronal death in PSN was recently shown (5). The re-evaluation of the lesions by light and electron microscopy revealed the typical morphological changes of apoptosis. Analysis of DNA fragmentation with an *in situ* tailing technique equally suggested an apoptotic mode of cell death in PSN (5, 46).

Apoptosis is a form of programmed cell death that is morphologically characterized by cell shrinkage, condensation of the chromatin, and its deposition at the inner surface of the nuclear membrane. As the process continues, the nucleus becomes fragmented and the cell breaks up into membrane-bound vesicles called apoptotic bodies (28). Programmed cell death is tightly regulated, and proteins promoting as well as inhibiting apoptosis have been identified (for review see [20]). Members of the bcl-2 family, such as bax and bcl-2 are involved in the regulation of cell death at the level of the mitochondria, where they may operate as ion channels. The relative abundance of pro- and anti-apoptotic members of the bcl-2 family is crucial for the cell's fate — life or death (55). p53 is a transcriptional regulator that induces apoptosis primarily after genotoxic insults and

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Age	Sex	Clinical data	Neuropathological diagnoses	Pathological diagnoses
39th week of gestation	F	intra-uterine death, preeclampsia	PSN	placental infarction, amnionic fluid aspiration
38th week of gestation	M	intra-uterine death	PSN	placental insufficiency
8 days	M	vitium cordis	PSN	1. aortic valvular stenosis 2. valvular mitral insufficiency 3. persisting Ductus Botalli
3 weeks	F	vitium cordis	PSN	1. hypoplastic left ventricle 2. atresia of the aortic valve and the ascending aorta 3. atresia of the mitral valve 4. hypertrophy of the right ventricle
1 month	M	connatal CMV infection	1. PSN 2. multiple focal dysplasias of the cerebellar cortex	1. connatal CMV infection 2. hepatosplenomegaly 3. open foramen ovale 4. bronchopneumonia
38th week of gestation	F	maternal HELLP-syndrome	PSN	1. fetopathia diabetica 2. disturbed placental maturity 3. amnionic fluid aspiration
6 weeks	M	hypotrophic immature infant of the 37th gestational week; vitium cordis	PSN	single ventricle with \ valvular mitral stenosis WPW-syndrome
3 weeks	M	vitium cordis, colitis	1. PSN 2. massive intracerebral hemorrhage 3. brain edema with single ventricle transtentorial herniation	1. single ventricle 2. D-transposition of the great arteries 3. persisting Ductus Botalli 4. interventricular septal defect 5. necrotizing enterocolitis
1 day	M	vitium cordis	1. PSN 2. multiple hemorrhages in the white matter	malformation syndrome with lung hypoplasia
39th week of gestation	M	stillborn	PSN	no cause of death found
2 weeks	M	vitium cordis, situs ambiguus, asplenia	1. PSN 2. periventricular infarct	1. single left ventricle 2. subvalvular pulmonary stenosis 3. atrial septal defect 4. situs ambiguus
1 month	M	vitium cordis	PSN	1. L-transposition of the great arteries 2. persisting Ductus Botalli 3. pulmonal stenosis
Control cases				
41th week of gestation	F	intrauterine death	no pathologic abnormality	compression of umbilical cord vessels
39th week of gestation	F	intrauterine death	no pathologic abnormality	n.a.
40th week of gestation	M	stillborn, perinatal asphyxia	no pathologic abnormality	amnionitis, aspiration pneumonia
39th week of gestation	n.a.	intrauterine death	no pathologic abnormality	n.a.

n.a. = not available

Table 1. Clinical and pathological data of PSN and control cases.

has also been reported to play a role in hypoxic-ischemic and excitotoxic neuronal death (7, 22). p53 induces Fas/Apo1, DR5, bax and other cell death related proteins and represses survival mediators such as IGF-1R (56). Programmed cell death finally culminates in an execution phase where key cellular structures undergo proteolytic digestion. Caspase-3/CPP32 appears to be one of the main executioner enzymes. Activated caspase-3 cleaves a wide range of proteins, among them structural proteins, regulatory proteins, and proteins involved in DNA repair (41)(52). A well established sequel of DNA damage is the activation of poly(ADP-ribose) polymerase (PARP) and the ensuing addition of poly(ADP-ribose) (PAR) to nuclear proteins (4). PARP is activated by free DNA ends generated during the apoptotic process, and its consumption of energetic substrates needed for poly(ADP-ribose) synthesis, *i.e.* NAD+, may lead to energy depletion and contribute to cell death (4). Increased poly(ADP-ribosyl)ation has been reported in extracts of apoptotic cells, and detection of PAR has been used as an early marker for apoptosis associated DNA damage (40).

In the present study, the pro-apoptotic factors p53, bax, caspase-3/CPP32 in its pro- and its activated form,

the anti-apoptotic protein bcl-2, and PAR as a marker of PARP activity were investigated in 12 cases of PSN. Immunohistochemistry as well as quantitative morphological evaluation was performed to elucidate the mechanisms of cell death in neuronal apoptosis in PSN.

Materials and Methods

Tissue. Pontine and hippocampal sections from 12 autopsy cases of PSN (38 weeks of gestation to 6 postnatal weeks) were investigated. From 8 cases, hippocampal as well as pontine tissue was available; from 3 cases, only pontine tissue and from 1 case only hippocampal tissue could be examined. The tissue was collected at the Department of Neuropathology of the University of Göttingen during the years of 1989-1994, formalin-fixed, and paraffin-embedded. The main clinical, neuropathological and pathological data are summarized in Table 1. Furthermore, pontine and hippocampal sections from 4 brains of age-matched infants without neuropathological disease (see Table 1) and especially lacking signs of PSN were used as control tissue for the expression of cell death associated markers at early developmental stages.

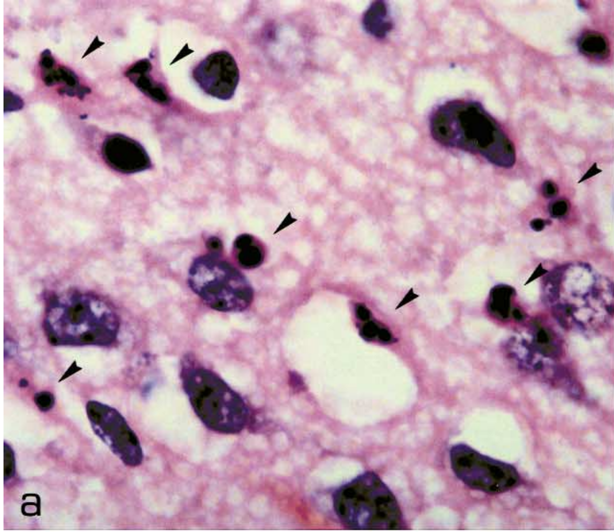
Neuropathology, immunohistochemistry, and detection of DNA fragmentation (TUNEL assay). Paraffin sections were dewaxed and stained with hematoxylin/eosin and luxol fast blue. Immunohistochemistry was performed on serial sections with primary antibodies against bcl-2 (Dako, Denmark; clone 124; 1:50), p53 (Pharmingen, CA, USA; clone DO-1; 1:20), bax (Pharmingen; clone G206-1276; 1:100), caspase-3/CPP32 (Dako; rabbit polyconal; 1:200), activated caspase-3 (CM-1; rabbit polyclonal; kindly provided by IDUN Pharmaceuticals, La Jolla, CA, USA; 1:5000), and poly (ADP-ribose) (Serotec, Oxford, UK; clone 10H; 1:300). The antiserum against caspase-3/CPP 32 recognizes procaspase-3 and the large subunit of the activated enzyme whereas CM-1 is specific for the large subunit and thus for activated caspase-3 (48, 49). Sections were pretreated with 3×5 minutes of microwaving in 10mM citric acid buffer pH 6.0 for antibodies against bcl-2, bax, caspase-3/CPP 32, and CM-1. Appropriate positive and negative control sections were included for individual antibodies. Sections processed with isotype control antibody or without primary antibody were used as controls and did not reveal any staining. Both alkaline phosphatase/anti-alkaline phosphatase (APAAP) and standard avidin-biotin based techniques were used. Neufuchsin and diaminobenzidine were used as chromogens. Sections were counterstained with hematoxylin. For the detection of fragmented DNA, terminal deoxynucleotidyl transferase (TdT)-mediated incorporation of digoxigenin-labeled nucleotides was used as described in detail earlier (17).

Morphometry. The number of apoptotic and immunostained neurons was determined in at least 8 standardized microscopic fields of 62, 500 μm^2 each, defined by an ocular morphometric grid, at a magnification of $\times 400$ in PSN and controls: *(1)* cells with the typical nuclear morphology of apoptosis, *(2)* cells with the typical nuclear morphology of apoptosis stained immunohistochemically with the markers mentioned above, and *(3)* cells stained by immunohistochemistry in the absence of the typical morphology of apoptosis were counted. Cell numbers were determined in the ventral pontine nuclei at the anatomical level of the locus coeruleus as well as in the subiculum at the level of the lateral geniculate body. Values represent the number of positive cells per square millimeter. For statistical analysis, the nonparametric Mann-Whitney U test was applied.

Results

Neuropathological findings in PSN. Between 45 and 405 cells/mm^2 in the subiculum and 14 and 363 cells/mm^2 in the pons revealed the key characteristics of apoptosis. Nuclei showed dense condensation of the chromatin that was sometimes found compacted at the nuclear membrane in a crescent shape. In the majority of cases, however, we detected the final stages of apoptosis with extensive nuclear fragmentation (Figure 1a). The presence of apoptotic cells with massive DNA fragmentation was confirmed by the TUNEL assay (Figure 1b). Cells with apoptotic morphology were predominantly seen in pons and subiculum, but also less frequently in areas of temporal cortex. Numbers of apoptotic cells were higher in the subiculum (203 +/- 41.7 cells/mm^2; all values +/- standard error (SE)) compared to the pons (125.4 +/- 34.9 cells/mm^2).

Expression of apoptosis related proteins in PSN. Immunohistochemistry for the anti-apoptotic marker bcl-2 revealed neuronal staining in temporal cortex laminae II-III. The majority of labeled neurons displayed regular morphology, although single bcl-2 positive apoptotic cells could be observed. In cases of PSN as well as in control brains, no bcl-2 positive cells were found in subiculum and pons except for scattered lym-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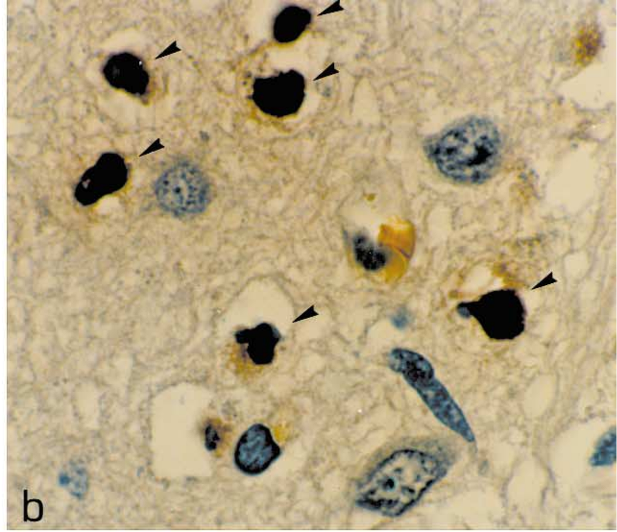
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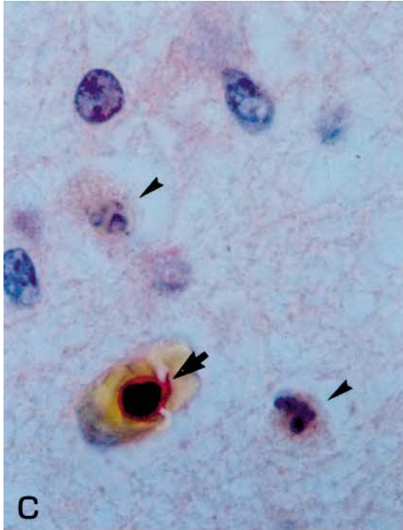
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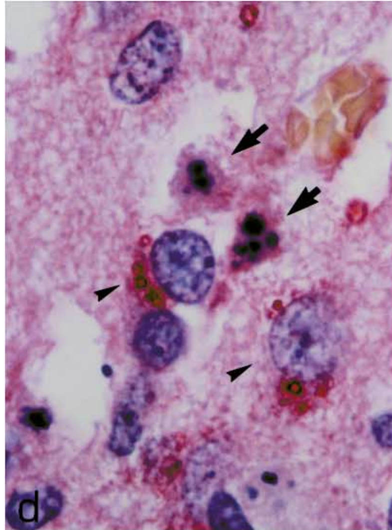
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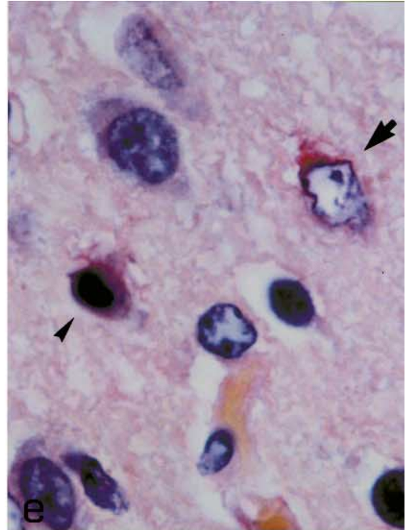
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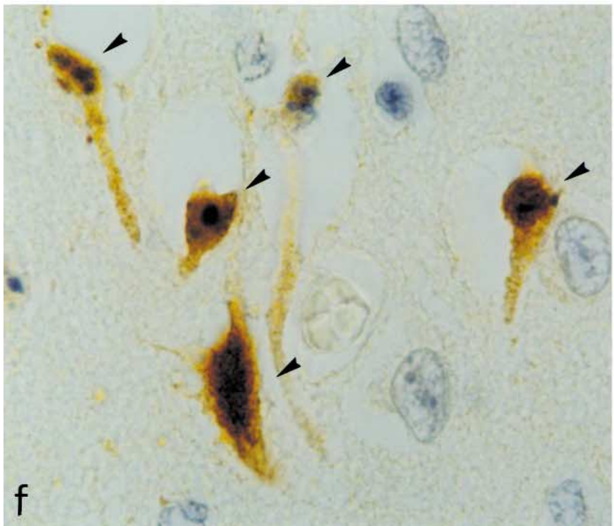
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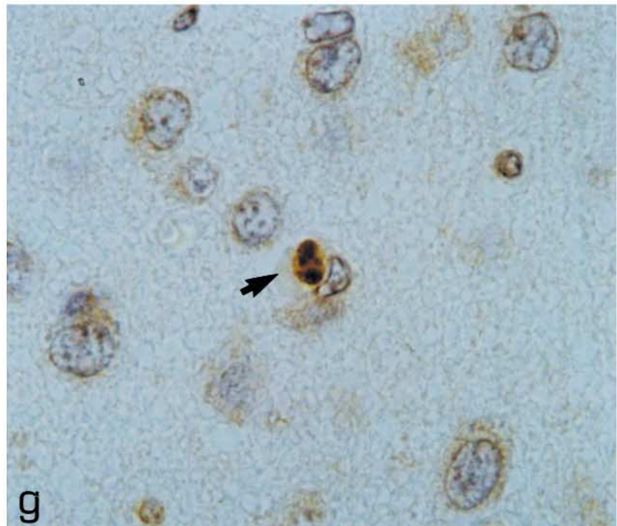
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pho-monocytic cells within vessels (Figure 1c). In our material of PSN and controls, no immunoreactivity for p53 could be found, although p53 protein was easily detected in lymphoma tissue serving as positive control.

In contrast, high numbers of bax-positive cells were detected in PSN brain (Figure 2a, b). The majority of neurons lacking morphological features of apoptosis stained positive. 207 +/- 49.3 neurons/mm^2 in the subiculum and 167 +/- 53.7 neurons/mm^2 in the pons showed bax immunoreactivity. Neuronal staining was either diffusely cytoplasmic or granular (Figure 1d). Furthermore, astrocytic cells showed a strong response with the bax antibody. However, only a subpopulation of cells with the typical apoptotic morphology revealed bax positivity (Figure 1d; Figure 2): the percentage of immunoreactive apoptotic cells was 1.2 +/- 0.8 % in the subiculum and 22.2 +/- 5.8 % in the pons (Figure 3). Similarly, in the control brains examined, the majority of normal appearing neurons was bax-positive: 688.6 +/- 149.5 neurons/mm^2 in the subiculum and 332 +/- 35.4 neurons/mm^2 in the pontine nuclei were stained. The number of bax-positive normal appearing neurons was significantly higher in controls than in cases of PSN ($p = 0.0264$ for the pontine nuclei and $p = 0.0028$ for the subiculum). Astrocytic staining was also observed in control brains.

Similar results — although less pronounced — were obtained with an antibody against procaspase-3/CPP32 (Figure 2a, b). Numbers of stained neurons not revealing the typical nuclear morphology of apoptosis were lower compared to bax ranging from below one neuron/mm^2 in the subiculum to 93 +/- 30.7 neurons/mm^2 in the pons. Cases with prominent staining of astrocytes were observed. However, only few cells stained with the anti-CPP32 antibody showed the typical nuclear morphology of apoptosis (Figures 1e, 2a, b). Similar to the expression of bax, the percentage of immunopositive apoptotic cells was lower in the subiculum (11 +/- 5.1 %) than in the pons (23.7 +/- 8.2%) (Figure 3). In control brains without PSN, hardly any neuron revealed procaspase-3/CPP32 expression in the subiculum. In contrast, similar to the results obtained in PSN, 310 +/- 32.3 pontine neurons/mm^2 were immunopositive. The number of normal appearing immunopositive neurons in controls was significantly higher compared to PSN ($p = 0.003$ for the pons).

Quite different results were obtained applying an antiserum against activated caspase-3 (Figure 2a, b). Almost all cells exhibiting the typical characteristics of apoptosis revealed immunostaining with the CM-1 anti-

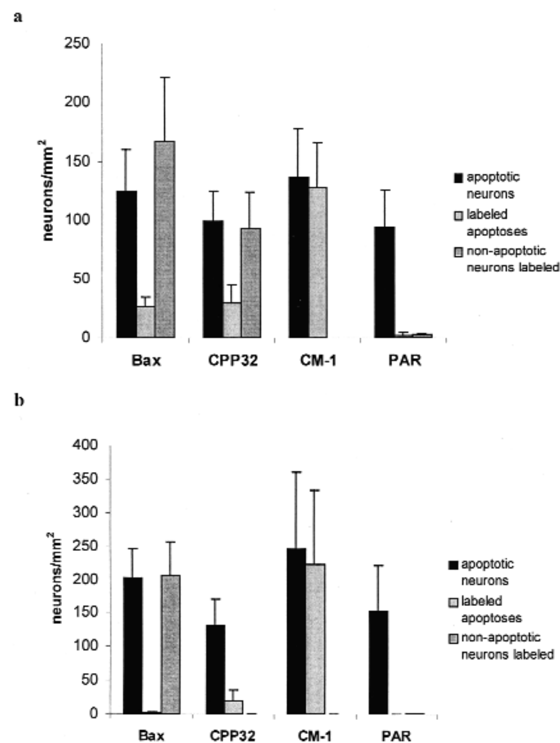

Figure 2. Incidence of immunoreactivity for bax, CPP32/procaspase-3, CM-1, and PAR in pontine and subicular neurons in PSN. **a.** In pontine sections, antibodies against bax and CPP32/procaspase-3 label a set of apoptotic as well as many normal appearing neurons. Labeling of CM-1 is restricted to apoptotic cells. Only few apoptoses reveal poly(ADP-ribosyl)ation. **b.** In the subiculum, almost no apoptotic neurons display bax immunoreactivity. CPP32/procaspase-3 is found in a limited number of apoptotic cells and hardly any normal appearing neurons. CM-1 staining is evident in the majority of apoptotic cells. PAR-immunoreactivity is nearly absent. Data are presented as mean ± standard error.

Figure 1. (Opposing page) **a.** Pontine apoptotic cells displaying hyperchromatic, condensed and often fragmented nuclei and eosinophilic, shrunken cytoplasms (arrowheads; H&E). **b.** Fragmented DNA in apoptotic cells is detectable using the TUNEL assay (black reaction product, arrowheads). **c.** Bcl-2 protein is not detected in apoptotic neurons (arrowheads); note the labeled intravascular inflammatory cell serving as internal positive control (arrow). **d.** Apoptotic (arrows) as well as normal appearing subicular neurons (arrowheads) showing bax immunoreactivity. **e.** Only few apoptotic subicular neurons are immunostained for CPP32/procaspase-3 (arrowhead); staining of astrocytes is observed occasionally (arrow). **f.** Subicular neurons labeled with the CM-1 antibody against activated caspase-3. Neuronal cell shapes and processes are easily discernable (arrowheads). **g.** A single apoptotic cell revealing poly(ADP-ribosyl)ation (arrow). **a-g:** original magnification: ×1000.

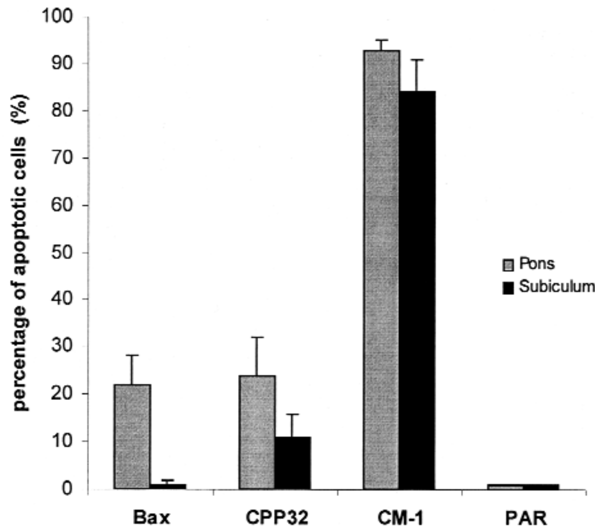

Figure 3. Relative number (%) of apoptotic cells stained for apoptosis-associated proteins in PSN brain. Bax and CPP32/procaspase-3 are found in around 20% of pontine apoptotic neurons and to a lesser extent in apoptotic subicular neurons. Close to 90% of apoptotic neurons contain activated caspase-3 as determined by immunohistochemistry applying the CM-1 antibody. Poly(ADP-ribosyl)ation was detected in less than 1% of apoptotic neurons in PSN. Data are presented as mean ± standard error.

body (Figure 1f). In 93.2 +/- 2.2 % of apoptotic pontine neurons and 86.4 +/- 6.9% of apoptotic subicular neurons, activated caspase-3 could be detected (Figure 3). Furthermore, CM-1 proved to be very specific for apoptotic cells in that only exceptional non-apoptotic cells, mainly reactive astrocytes, were faintly labeled. In control cases, no labeling was observed except for single scattered apoptotic cells.

Poly(ADP-ribosyl)ation — as detected by the anti-PAR antibody — was restricted to a minor proportion of apoptotic neurons (Figures 2a, b, 3). 2.3 +/- 2.8 cells/mm^2 with unequivocal apoptotic morphology were stained in the pons. Staining was predominantly nuclear and involved cells with explicit nuclear fragmentation (Figure 1g). In the subiculum, below 1 cell/mm^2 revealed PAR-immunoreactivity. Rarely, highly condensed nuclei revealed PAR-staining, where it was impossible to unequivocally determine apoptotic morphology. The number of PAR-positive apoptotic neurons varied considerably between cases. Apart from the intense labeling of apoptotic nuclei, diffuse light cytoplasmic staining of the majority of large pontine or subicular neurons was observed in PSN as well as in control cases and not counted. In control cases, only exceptional nuclear PAR-immunoreactivity was observed.

Discussion

Pontosubicular neuron necrosis (PSN) is a type of perinatal brain damage showing the typical characteristics of apoptosis predominantly in pontine and subicular neurons (5). Most infants with PSN suffer from cardiac malformations or pulmonary diseases suggesting that prolonged hypoxia-ischemia is capable of inducing apoptosis in certain neuronal subpopulations within a critical time window (5, 13). However, the underlying mechanisms leading to widespread apoptotic cell death in PSN are unknown, and the expression of pro- and antiapoptotic proteins in the neuronal subpopulations involved has not been examined. In our study, we investigated a panel of pro- and antiapoptotic proteins including p53, bax, bcl-2, and caspase-3 and studied the extent of poly(ADP-ribosyl)ation in cases of PSN and age matched controls. Our results indicate that caspase-3 is activated in the vast majority of apoptotic pontine and subicular neurons and proves a reliable and sensitive marker for the detection of apoptosis (49).

Caspase-3/CPP32 is one of several central effector caspases that integrate various cell death signals and initiate the cleavage of key cellular substrates, such as gelsolin, fodrin, actin, focal adhesion kinase, and PARP (poly(ADP-ribose) polymerase). Caspases thereby destroy important structural components and disable critical homeostatic and repair processes, thus resulting in the disassembly of the cell (41). Caspase-3 is activated during apoptosis in many types of neurons including cortical neurons (27) and cerebellar granule cells (1). However, models and pathways of caspase-3 independent apoptotic cell death have been described (8, 50), and activation of caspase-3 has not been observed in neurons dying by necrosis (1). In our study, the catalytically inactive enzyme procaspase-3 was readily detected in normal appearing pontine neurons in both cases of PSN as well as in controls suggesting constitutive expression. In contrast, procaspase-3 was only rarely found in healthy subicular neurons. Despite this difference, during apoptosis, activated caspase-3 was detectable in both pontine and subicular neurons with similar frequency raising the possibility that levels of subicular procaspase-3 are subject to rapid upregulation after apoptosis induction or below the immunohistochemical detection level in our histological material. Our results reveal a nearly 100% correlation between cells showing the typical nuclear morphology of apoptosis and staining with the CM-1 antibody against active caspase-3. This indicates activation of caspase-3 in neuronal apoptosis in PSN and suggests that caspase-3 is a major effector caspase in this disease. The high concordance

of CM-1 immunopositivity with classical apoptotic morphology in PSN and other paradigms suggests that activation of caspase-3 is crucial for the morphological features of apoptosis (29, 48, 49). Furthermore, detection of activated caspase-3 may serve as a specific tool to detect apoptotic cells *in vitro* and in tissue sections.

Members of the bcl-2 family play a key role in determining the propensity of a cell to die in response to stressors. Subtle shifts in the ratio between pro- and antiapoptotic molecules may alter a cell's life-death balance (55). Increased bax expression is associated with neuronal apoptosis in various experimental paradigms, such as kainic acid induced seizure (16), cortical focal ischemia (25), and cardiac arrest (26). However, increased immunostaining for bax after a nervous system injury is not universal, as bax protein levels decline in the infarcted caudate and putamen after permanent occlusion of the middle cerebral artery in rats (14). We found that in PSN and controls, most pontine and subicular neurons expressed bax constitutively. The majority of these bax positive neurons did not show an apoptotic morphology. Our failure to detect bax in many apoptotic neurons may be related to the presumed conformational change of bax upon association with mitochondria that our antibody may be unable to detect (9). Abundant evidence exists that bcl-2 is capable of inhibiting ischemic neuronal cell death (38). We did not detect any neuronal bcl-2 expression in pontine and subicular neurons in cases of PSN or controls suggesting that during early CNS development, bcl-2 is not the major anti-apoptotic protein in the neuronal populations examined. Instead, bcl-x_L has been reported to be upregulated during early stages of neuronal development and most likely functions as an important anti-apoptotic regulator at the time points studied (18, 42, 57).

The detection of poly(ADP-ribosyl)ated proteins has been used to identify apoptotic cells (40). Increased levels of poly(ADP-ribosyl)ated proteins have been detected in brain ischemia and Alzheimer's disease (33, 34). Poly(ADP-ribose) polymerase (PARP) is activated by DNA strand breaks and catalyzes the addition of poly(ADP-ribose) to nuclear proteins (44). Massive activation of PARP may lead to cell death due to energy depletion (4, 58). In our material, only few apoptotic neurons contained poly(ADP-ribosyl)ated proteins suggesting that PARP is not regularly activated during neuronal apoptosis in PSN whereas it has been described to be markedly expressed in another study (37). This difference may be explained by the use of different reagents and the fact that poly(ADP-ribosyl)ated proteins may only be detectable during a short time period.

p53 is influencing cell death pathways via transcriptional and non-transcriptional mechanisms (56). A role for p53 in the regulation of cell death in various models of hypoxic-ischemic and excitotoxic neuronal death has been described (15, 22, 31). A role for p53 in cell survival and DNA repair after ischemia has also been discussed (53). While we were able to detect p53 in lymphoma, we did not detect immunoreactivity for p53 in either PSN or in controls suggesting that initiation and progression of apoptosis in PSN is p53-independent.

In the fetal and neonatal brains with PSN, hypoxic-ischemic injury leads to massive apoptotic neuronal cell death of selective neuron populations. A similar observation has been made in neonatal rodents subjected to experimental hypoxia-ischemia (6, 43). In contrast, ischemia in the adult brain is dominated by necrotic cell death, and apoptosis is predominantly seen in the penumbra, where tissue damage is less severe (21). It is currently a matter of debate whether apoptotic cell death contributes substantially to adult ischemic neuronal death, mainly because the morphological hallmarks of classical apoptosis are in general not conspicuous in human autopsy brains (10, 21, 35, 45). Since both neonatal and adult rodent ischemic neuronal death is sensitive to caspase inhibition (11, 23, 32), the designation "caspase-dependent cell death" for ischemic cell death has been proposed (3, 45). While the morphological appearance of caspase-dependent cell death differs between neonatal and adult hypoxic ischemia, caspase inhibitors are effective in blocking ischemic cell death and reducing infarct size in both situations (32), and may therefore prove valuable therapeutic agents (6, 11, 23). It remains to be determined why neurons in the adult nervous system react differently in response to hypoxic-ischemic damage. Differences in the expression of pro- and antiapoptotic proteins and in the regulation of energy metabolism may play a role (30). Similar cellular mechanisms may underly the phenomenon that in the adult CNS, ischemia and other insults induce apoptotic as well as necrotic cell death in distinct neuronal populations (2, 19, 36, 47). In this context, PSN represents a useful model of caspase dependent neuronal apoptosis in which the molecular regulation of cell death can be studied *in situ* in human CNS tissue.

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