RESEARCH ARTICLE

Neuronal Apoptosis in the Dentate Gyrus in Humans with Subarachnoid Hemorrhage and Cerebral Hypoxia

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(For a perspective on cell death mechanisms, see article by Graeber & Moran, p. 385)

Apoptosis of dentate granule cells is a typical feature of several animal models of disease. In 20 autopsy cases of subarachnoid hemorrhage (SAH) and global cerebral hypoxia caused by protracted shock or respiratory failure, we evaluated by light microscopy and in situ tailing whether this pattern of neuronal damage also occurs in humans. In subarachnoid hemorrhage, 4.0/mm² (0-13.0/mm²) apoptotic neurons were observed in the dentate gyrus, in cerebral hypoxia 3.6/mm² (0-19.9/mm²) (p>0.05), and in 10 aged-matched control cases dying rapidly from non-neurological diseases 0/mm² (0–0/mm²) (median [range]) (p<0.001 versus SAH and hypoxia). Neuronal apoptosis in the dentate gyrus was most frequent, when death occurred later than 24 hours and less than 11 days after disease onset. Neuronal damage in the hippocampus was always necrotic. It was more severe in hypoxia than in SAH (median neuronal damage score 3 [range: 0-3] versus 0 [0 - 3], p<0.001).

Apoptosis appears to be the predominant mechanism of death in dentate granule cells irrespective of the underlying disease, whereas neuronal death in the hippocampus generally is of necrotic morphology.

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Introduction

Apoptosis of neurons in the granule cell layer of the dentate gyrus is a frequent observation in animal models of disease (adrenalectomy [52, 53], focal ischemia [29, 33], generalized hypoxia [44], epileptic seizures [1, 11, 12], traumatic brain injury [9, 25], radiation [38, 42], meningitis [6, 28, 60]), and in humans dying from bacterial meningitis (13, 40).

The hippocampal formation plays an important role in memory storage during learning (50, 55, 59). After experimental traumatic brain injury, posttraumatic memory scores were correlated with neuronal loss in the hilus of the dentate gyrus in rats (19). In the absence of other hippocampal abnormalities, apoptosis of dentate granule cells produces learning impairment in the radial arm and Morris water maze, which test spatial learning and memory (10, 55). In humans, neuropsychological evidence points to an impaired function of the hippocampal formation after subarachnoid hemorrhage and global cerebral hypoxia (21, 46). The present study was performed to evaluate whether apoptotic neuronal damage in the dentate gyrus is a characteristic common feature of severe neurological disease.

Materials and Methods

Patients dying after subarachnoid hemorrhage and cerebral hypoxia between 1995 and 1999, who underwent autopsy, were identified retrospectively by reviewing the records of the Department of Neuropathology, Georg-August-Universitiy, Germany. Twenty brains of patients dying from subarachnoid hemorrhage (12 women, 8 men, age 3-84 years, median = 44 years, 18 spontaneous, 2 post-traumatic events) and 20 brains of patients dying of protracted diseases causing cerebral hypoxia (9 women, 11 men, age 17-85 years, median = 60 years) were evaluated in the present study. Diagnoses in the hypoxia group were cardiogenic shock (n = 8), septic shock (n = 3), hemorrhagic shock (n = 1), cause of shock and multiorgan failure not specified (n = 5), death subsequent to carbon monoxide intoxication (n = 1), and respiratory failure (n = 2). The brains of 10 patients (6 women, 4 men, age 1 month-77 years, median = 38.5 years) without abnormalities on routine brain examination served as controls. These patients had died rapidly from non-neurological diseases (3 sudden cardiac deaths, 2 suffocations, 2 suicides, 1 overwhelming post-splenectomy infection, 1 drowning, 1 sudden infant death). The interval between death and onset of symptoms of the underlying disease was 0 to 33 days (median = 1 day) in subarachnoid hemorrhage, 0 to 52 days (median = 2.5 days) in global cerebral hypoxia, and 0 to

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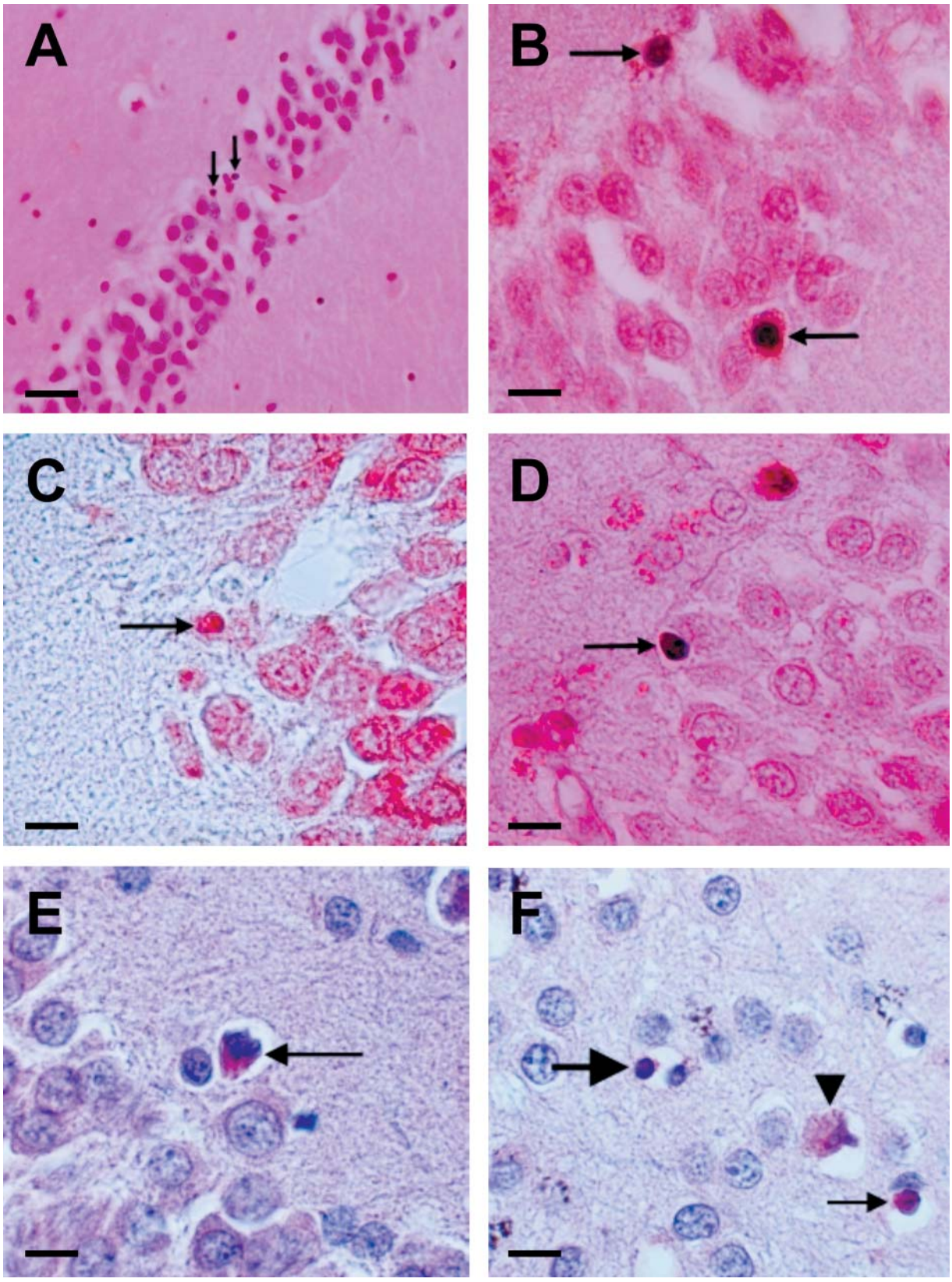
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1 day (median <1 day) in control patients. In patients dying from SAH and hypoxia, autopsy was performed 12 to 96 hours (median = 24 hours) and 12 to 120 hours (median = 24 hours) after death. In control cases, the interval between death and autopsy ranged from 24 to 96 hours (median = 48 hours). The study was approved by the Ethics Committee of the University Hospital of Göttingen.

Histology. Paraffin sections (1 μm) of the hippocampal formation were stained with hematoxylin-eosin (HE) for the morphological assessment of neuronal damage. Routine histological examination (HE) was performed on different brain regions including neocortex, basal ganglia, cerebellum and brain stem.

In situ tailing (IST). Deparaffinized and hydrated 1 μm sections (1 per case) were treated with 50 μg/ml proteinase K (Sigma, Deisenhofen, Germany) for 15 minutes at 37°C. The sections were incubated for 1 hour at 37°C in a reaction mixture containing 10 μl of 5× tailing buffer, 1 μl digoxigenin DNA labeling mix, 2 μl cobalt chloride, 12.5 U terminal transferase and the necessary amount of distilled water to give a volume of 50 μl. After washing, the sections were incubated with 10% fetal calf serum (FCS) for 15 minutes at room temperature. A solution of alkaline phosphatase-labeled anti-digoxigenin antibody in 10% FCS (1:250) was placed on the sections for 60 minutes at 37°C. The color reaction was developed with 4-nitroblue-tetrazolium-chloride/5-bromine-4-chloride-3-indolyl-phosphate (NBT/BCIP). The sections were counterstained with nuclear fast red-aluminium hydroxide. All reagents were purchased from Boehringer Mannheim, Germany.

Quantification of apoptotic neurons. HE-stained sections were used to measure the area of the granule cell layer with a Contron videoplan computer (Grundig, Nürnberg, Germany). Apoptotic cells were counted in the adjacent section stained with the IST reaction. The same cells showed morphologic features and IST staining indicating apoptosis. The density of apoptotic neurons was expressed as the number of marked neurons per mm^2 of the granule cell layer (13, 40, 60).

Immunohistochemistry. Selected paraffin sections were stained by immunohistochemistry using a mouse monoclonal anti-NeuN antibody (MAB 377, Chemicon, Temecula, Calif) as primary antibody (dilution 1:100) to confirm the neuronal nature of the apoptotic cells (Figure 1c). Activated caspase-3 was detected by the rabbit polyclonal anti-human antibody CM-1 (kindly provided by IDUN Pharmaceuticals, La Jolla, Calif, dilution 1:3500) (Figures 1e, f). The antibodies were used in conjunction with the alkaline phosphatase/ anti-alkaline phosphatase technique, and the color reaction was developed with Newfuchsin (all reagents purchased from Dako, Kopenhagen, Denmark). Sections stained with the CM-1 antibody were counterstained with hemalum.

Hippocampal necrosis score. On HE-stained sections, the density of necrotic neurons in the areas CA1 to CA4 of the hippocampus was assessed as absent (0), <10% of all neurons (1), 10 to 30% of all neurons (2), and >30% of all neurons (3).

Statistical analysis. Because of the heterogeneity of cases, an explorative statistical analysis was performed. The nonparametric 2-tailed Kruskall-Wallis test was used to compare the SAH, hypoxia and control group. Dunn's Multiple Comparisons Test was used as post-test to correct for repeated testing, when the overall p was <0.05. Two-tailed Spearman's rank correlation coefficient was employed to study the relation between the hippocampal injury score and the intervals "onset of the disease→death" and "death→autopsy" versus the density of apoptotic neurons in the dentate gyrus. P values <0.05 were considered significant.

Results

Neuronal apoptosis in the granule cell layer of the dentate gyrus was identified using 2 different criteria: in HE-stained sections, the cells demonstrated condensation

Figure 1. (Opposing page) Neuronal apoptosis in the dentate gyrus of humans dying from subarachnoid hemorrhage and cerebral hypoxia. *Subarachnoid hemorrhage*: HE staining (**a**) (×220), in-situ tailing (**b**) (×1100), and NeuN immunohistochemistry (**c**) (×1100) in a 53-year-old woman dying of spontaneous subarachnoid hemorrhage. *Cerebral hypoxia*: In situ tailing (**d**) (×1100) in a 74-year-old woman dying from cardiogenic shock after myocardial infarction. Apoptotic neurons are marked by arrows. *Immunohistochemistry for activated caspase-3*: (**e**) Dentate granule cell with a condensed, shrunken nucleus and narrow cytoplasm (arrow), septic shock. 32-year-old man (×1100). (**f**) Neuron with apoptotic morphology (small arrow) and with early signs of injury (arrowhead) staining positive for activated caspase-3. Strong cytoplasmic shrinkage impeding the detection of activated caspase-3 in the cytoplasm in late stages of apoptosis (large arrow). Subarachnoid hemorrhage, 66-year old woman (×1100). Space bars: (**a**) 50 μm, (**b-f**) 10 μm

	SAH	Hypoxia	Controls	p*
n	20	20	10	
Age	44 (3-84)	60 (17-85)	38.5 (0.1-77)	0.14
Sex (m/f)	8/12	11/9	4/6	0.64
Interval between onset of disease and death (Hours)	1 (0-33)	2.5 (0-52)	<1 (0-1)	0.003 SAH vs. Co<0.01 Hypoxia vs. Co<0.01 SAH vs. hypoxia>0.05
Interval between death and autopsy (Hours)	24 (12-96)	24 (12-120)	48 (24-96)	0.08
Apoptotic neurons in the dentate gyrus (mm^{-2})	4.0 (0-13.0)	3.6 (0-19.9)	0 (0-0)	<0.0001 SAH vs. Co<0.001 Hypoxia vs. Co<0.001 SAH vs. hypoxia>0.05
Hippocampal necrosis score	0 (0-3)	3 (0-3)	0 (0-2)	<0.0001 SAH vs. Co>0.05 Hypoxia vs. Co<0.001 SAH vs. hypoxia<0.001

Table 1. Patients dying from subarachnoid hemorrhage (SAH) and cerebral hypoxia. *Kruskall-Wallis nonparametric analysis of variance. Post-tests were performed (Dunn's Multiple Comparisons Test), when p of the overall test was <0.05.

of the nuclear chromatin and shrinkage of the cytoplasm (Figure 1a). DNA fragmentation within apoptotic cells was visualized by in situ tailing (SAH: Figure 1b; hypoxia: Figure 1d).

Neuronal apoptosis was observed in the granule cell layer of the dentate gyrus in 16 of 20 patients dying from SAH, 19 of 20 patients dying after cerebral hypoxia, and in none of the control brains after sudden death. The median (minimum-maximum) density of apoptotic neurons in the dentate gyrus was 4.0 (0-13.0)/mm^2 in SAH, and 3.6 (0-19.9)/mm^2 in hypoxia patients (difference not significant).

When each disease group was analyzed separately, no clear relation between the density of apoptotic neurons and the interval between the onset of symptoms of disease and death was noted. Only when patients of all groups were analyzed together, the density of apoptotic neurons depended on the interval between onset of disease and death ($p = 0.005$): *i)* in patients dying less than 24 hours after the onset of disease, in median 0 (range: 0-8.9) neuronal apoptoses/mm^2 occurred; *ii)* the highest density of 3.90 (0-19.9) apoptotic neurons/mm^2 was observed in patients dying more than 24 hours and less than 11 days after the onset of the disease ($p<0.01$ versus group [1]); and *iii)* patients dying after the acute phase (11 days and later after onset of symptoms), in median, had a lower density of apoptotic neurons in the dentate gyrus than patients dying from day 2 to 10 (2.1/mm^2 [0-5.0/mm^2], $p>0.05$ versus groups [1] and [2]). Approximately 20% of apoptotic neurons stained positive for activated caspase-3 (Figure 1e, f). Some neurons with beginning nuclear shrinkage, but without the morphological features of fully developed apoptosis, also stained positive for activated caspase 3 (Figure 1f), whereas morphologically intact neurons were negative (Figure 1e, f).

The density of neuronal apoptoses in the dentate gyrus did not correlate with age (SAH: $r_s = 0.11$, $p = 0.50$; hypoxia: $r_s = -0.07$, $p = 0.78$). In SAH and hypoxia, a weak negative correlation was found between the interval between death and autopsy and the density of apoptotic neurons in the dentate gyrus ($r_s = -0.30$, $p = 0.06$).

Neuronal damage in the hippocampus was of necrotic morphology. No apoptotic neurons were detected in the hippocampus. Necrotic pyramidal cells did not stain with the protocol used for in situ tailing in this study. After prolonged incubation in the tailing or color reaction mixture necrotic neurons were also labelled (14). Necrosis of hippocampal pyramidal cells with

eosinophilic degeneration of the cytoplasm and nuclear pyknosis was more severe in hypoxia than in SAH (median [minimum-maximum] 3 [0-3] versus 0 [0-3], $p<0.001$). The presence or absence of neuronal necrosis in the pyramidal cell layer of the hippocampus was not related to neuronal apoptosis in the dentate gyrus (SAH: $r_s = -0.09$, $p = 0.70$; hypoxia: $r_s = 0.10$, $p = 0.68$).

In the dentate gyrus of the 10 control cases, no neuronal apoptoses were detectable by HE staining and in situ tailing. In one of these cases, moderate necrotic neuronal damage was observed in the area CA1 of the hippocampus. The other brains showed no morphologic abnormalities.

Discussion

In recent years, the mistaken equivalence of the terms "apoptosis" and "preventable cell death" has caused confusion (51). Apoptosis of cells is characterized by involutional changes, in particular cell shrinkage. As in the present study, apoptosis usually affects single cells dispersed within an organ. On the opposite, necrosis involves cell swelling, accompanied by membrane breakdown, and frequently occurs in groups of cells simultaneously (20). Morphology of apoptosis consists of nuclear and cytoplasmic shrinkage, chromatin condensation and formation of apoptotic bodies. The biochemical features of apoptosis include endonucleolytic degradation of DNA into nucleosomal fragments, dependency on macromolecular synthesis and expression of apoptosis-specific proteins (8, 15, 20). IST alone to document apoptosis is unreliable, because fragmentation of the nuclear DNA can also occur in later stages of necrosis without the morphology of apoptosis (15, 16, 24, 30). Combined with morphological criteria, however, IST is a reliable method of detecting apoptosis (7, 8, 15, 37, 40). With the present protocol, IST labelled apoptotic granule cells, but not necrotic pyramidal cells. The probable reasons for the lack of IST staining of necrotic neurons were: *i)* the NBT/BCIP color reaction was stopped early to reduce non-specific labelling of cells not displaying the morphology of apoptosis. Non-specific staining sometimes occurred in glial cells; and *ii)* in necrosis, DNA damage is a late event. Histological abnormalities precede DNA degradation, leaving many cells of necrotic morphology unlabeled by the IST reaction (14, 15, 24, 30).

In this study, apoptosis was confined to the granule cell layer of the dentate gyrus. Compared to animal models of diseases (6, 9, 28, 29, 38, 42, 44, 60), the density of apoptotic granule cells was relatively low. This may be explained by the protracted course of the underlying disease of many of the autopsy cases studied and/or the rapid removal of apoptotic neurons by phagocytes (20, 23). Another reason may be true differences of the amounts of apoptotic granule cells in different diseases, eg, cerebral hypoxia in adults versus perinatal hypoxia (7).

Neuronal damage in the hippocampus and neocortex was necrotic. A similar pattern of neuronal injury in the hippocampal formation (apoptosis of dentate granule cells, necrosis of hippocampal pyramidal cells) occurs in several animal and cell culture models of disease (1, 9-11, 17, 25, 28, 29, 33, 36, 38, 42, 44, 49, 57, 60) and in human meningitis (40): In brains from rats subjected to 8 or 12 minutes of forebrain ischemia and 48 hours of reperfusion, neuronal death in the dentate gyrus was of apoptotic morphology (29). In an adult mouse model of hypoxia, in spite of labelling by the TUNEL reaction, morphological changes of degenerating CA1 neurons "were clearly distinct from apoptosis," whereas, "a significant amount of degenerating dentate granule cells...had typical apoptotic nuclei" (57). In a dog model of transient global incomplete cerebral ischemia, the ultrastructure of degenerating dentate and cerebellar granule cells was apoptotic, and the morphology of degenerating CA1 pyramidal neurons and cerebellar Purkinje cells necrotic (33). Hippocampal kindling stimulation and kainic acid-induced seizures caused apoptotic death of dentate neurons predominantly on the hilar border of the granule cell layer and in the polymorphic region (1). After X-ray irradiation (total dose: 10 and 18 Gy), rats and mice developed neuronal apoptosis exclusively in the granule cell layer of the dentate gyrus (38, 42). In organotypic hippocampal cultures from newborn rats and mice challenged with bacterial products (36, 49), apoptosis predominantly of granule cells in the dentate gyrus and necrosis preferentially of hippocampal neurons coexisted.

Cell death following target deprivation, excitotoxicity and ischemia can coexist as apoptosis, necrosis, and hybrid forms along an apoptosis-necrosis continuum. Whether apoptosis or necrosis predominates, depends on the noxious agent, the strength of the injury, the cell population involved and maturity of the cells (32, 47). Apoptosis and necrosis may share common initiation pathways and can be elicited by the same stimuli at different intensities (4, 17, 31). Rapid neuronal death frequently is necrosis, whereas delayed neuronal injury tends to be apoptosis (20, 41). Caspases provide a valuable tool for monitoring the mechanisms of neuronal loss. In many systems, the appearance of activated caspase-3 is strongly associated with morphological features

of apoptosis (58). Caspase-3 plays a major role in cell death in immature neurons, but a minor role in the death of mature neurons (22). In the present study, morphologically intact neurons did not contain activated caspase-3 as demonstrated by immunohistochemistry. As in meningitis (13), approximately 20% of apoptotic neurons stained positive for activated caspase-3. For several possible reasons, we were unable to document activated caspase-3 in all apoptotic neurons: *i)* cytoplasmic shrinkage impeded the detection of activated caspase-3 in the cytoplasm in late stages of apoptosis (Figure 1f, large arrow); *ii)* caspase-3 independent apoptosis may be involved (6); and *iii)* the relatively long intervals between death and autopsy may disturb caspase-3 immunohistochemistry.

In SAH and hypoxia, patients dying within the first 24 hours or later than 10 days after the onset of symptoms tended to show lower densities of apoptotic neurons in the dentate gyrus than patients dying during the acute phase of SAH and hypoxia. This trend attained statistical significance only, when all patients irrespective of their underlying disease were analyzed together. In the control subjects of the present study who died from rapidly fatal non-neurological diseases (maximum interval between onset of the disease and death 1 day), no apoptotic neurons were detectable in the dentate gyrus by morphology and in situ tailing. A similar time course of neuronal injury was observed in animal models (5, 24, 39, 41, 44) and in humans dying from bacterial meningitis (40).

As outlined above, various pathological conditions produce dentate granule cell apoptosis. Several mechanisms appear to be ultimately involved in the generation of neuronal damage: stimulation of glucocorticoid receptors (18), excitatory amino acids (54), cytokines (48) and oxygen radicals (26). In experimental subarachnoid hemorrhage, subarachnoid hemolysates appear to induce apoptotic neuronal damage (34, 35). Neuronal injury was less pronounced in mice over-expressing CuZn-superoxide dismutase suggesting an involvement of free radicals (34). Oxidative injury, excitotoxicity and activation of caspases can be linked closely: stimulation of neurons with excitatory amino acids leads to the production of reactive oxygen species (ROS) (27, 45), exposure of the hippocampus to ROS generating substrates leads to the release of excitatory amino acids (43), and glutamate uptake into cortical astrocytes is inhibited by ROS (56). Pro-caspase-3 can be cleaved to activated caspase-3 by m-calpain, which is activated by calcium influx during excitotoxicity (3). This implies that these mechanisms may potentiate each other.

Whether apoptosis of dentate granule cells is present only in persons dying after cerebral hypoxia or SAH or also occurs in survivors, being one cause of long-term neuropsychological deficits, remains unclear. Scintigraphy with radiolabeled annexin V may resolve this question (2).

In conclusion, apoptosis of granule cells in the dentate gyrus, which has been observed in several animal models of disease, occurs in patients dying from subarachnoid hemorrhage (SAH), hypoxia and bacterial meningitis. In the present study, the density of apoptotic dentate granule cells was almost identical in SAH and hypoxia and compared well with the rate of apoptotic granule cells observed in a previous autopsy study of bacterial meningitis (40). Human and animal data indicate that apoptosis of dentate granule cells may be a common pattern of neuronal death in various diseases with different pathogenesis. Neuronal damage in the pyramidal cell layer of various areas of the hippocampus was of necrotic morphology. This suggests a predilection of granule cells for apoptotic death, whereas pyramidal cells in adults usually die by necrosis.

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