Physiol. Res. 55: 339-348, 2006

Temporal Profile of Ultrastructural Changes in Cortical Neurons after a Compression Lesion

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Received October 25, 2004
Accepted July 18, 2005
On-line available August 5, 2005

Summary

We studied the occurrence of apoptosis and secondary delayed cell death at various time points in the *penumbra* zone, which is the target for therapeutic intervention after stroke. A compression lesion was induced in the right sensory motor cortex of rat brains. At 0.5, 1, 3, 6, 12, 24, 48 and 72 h after lesioning, motor functions were evaluated by behavioral tests, and cortical layers IV and V were examined by electron microscopy. Behavioral recovery was observed at 48 h after lesioning. At 0.5-1 h in the lesioned area, the neuropil was expanded and contained affected cells. Apoptotic cells were found between 0.5-72 h, and at 12 h, 47.3 % of the total cell number was apoptotic cells. On the contralateral side, cells showed an enlarged endoplasmic reticulum at 3 h, indicating secondary delayed cell death. Our results show that a compression lesion is a useful model for studying ultrastructural changes in injured cells. The lesion results in the *penumbra* zone with apoptotic cell death between 0.5-72 h. As secondary delayed cell death occurred on the contralateral side at three hours after lesioning might be the time period during which injured, but still viable, neurons can be targets for acute treatment.

Key words

Stroke • Ischemia • Sensory motor cortex • Cell death • Ultrastructure

Introduction

Neurological impairment and recovery after stroke depends on the lesion extent, its location and duration. To limit the extent of the lesion, neuroprotective drugs and thrombolysis with tissue plasminogen activator are useful acute treatments (Chopp *et al.* 1999, De Keyser *et al.* 1999a, 1999b, Zhang *et al.* 2001, Kidwell *et al.* 2002). However, no neuroprotective drug has proven to be effective as acute treatment in clinical trials (De Keyser *et al.* 1999b). Recent clinical results have shown that only 2-3 % of the patients benefit from thrombolytic treatment in early stroke within three hours after symptom onset. Many animal models fail in mimicking the acute stroke situation therefore treatments that were useful under experimental conditions might give unsatisfactory results in clinical trials (Dirnagl *et al.* 1999).

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Transient acute ischemia is well mimicked by a compression lesion model resulting in the *penumbra* zone (Kundrotiené *et al.* 2002). The *penumbra* zone of ischemic brains is defined by different criteria. Defined in static terms, the zone is a cellular interface between the ischemic cells in the core of the infarction, which are committed to die, and the unaffected cells in areas with normal blood flow (Hakim 1998). Defined as a zone of incomplete cerebral ischemia, where neurons are functionally inactive but still viable, the area is explained in biochemical terms (De Keyser *et al.* 1999a, 1999b). The literal translation ”half shadow” of the Latin word *penumbra* perhaps best describes the area.

In some studies, apoptosis, i.e. programmed cell death, has been suggested to be the main cell death pathway in the *penumbra* zone (Lee *et al.* 1999). However, necrosis and apoptosis are probably triggered in parallel, and furthermore, the apoptotic pathway has been suggested to lead to necrosis (Lipton 1999). The morphological criteria for necrosis and apoptosis are well defined. Necrosis is accidental cell death that results entirely from circumstances outside the cell and is manifested by karyolysis and cell swelling (Studzinski 1999). Apoptosis refers to a process that involves an inherent cellular program leading to cell death and which appears with karyorrhexis and pyknosis (Studzinski 1999). The apoptotic process is energy consuming and can be initiated by internal signals or by agents in the environment (Kerr *et al.* 1972).

Secondary delayed cell death occurs after a brief trauma, not directly within a short time but after a substantial time delay. The delay is between some hours and several days (Kermer *et al.* 1999). The morphological criteria are enlarged stacks of endoplasmic reticulum (ER), small dense bodies and the formation of large vacuoles (Lipton 1999). These structural changes usually occur in neurons surviving the primary insult and accumulate over time before the cells finally die. The affected neurons could, due to the time delay involved, be the targets for neuroprotective interventions (Kermer *et al.* 1999).

In this study we examined the ultrastructure of affected cells following incomplete ischemia in rat brains and correlated the results with motor functions. A transient focal compression lesion, well defined in time and space, represented incomplete ischemia in the *penumbra* zone. To identify the ultrastructural criteria of cell death, i.e. ruptured membranes and flocculent mitochondria, electron microscopy (EM) was used. Motor function was studied by examining sensory motor ability after lesioning in a beam-walking test with a 7-point rating scale (Feeney *et al.* 1981).

We aimed to study the occurrence of apoptosis and secondary delayed cell death at various time points after lesioning. To define therapeutic time windows, the time course of morphological features in affected neurons was correlated with motor function ability. During time periods in which neurons are affected but still viable, they are the main targets for acute intervention in cerebral ischemia.

Methods

Experimental design

This study was performed according to animal care legislation and approved by the Animal Ethical Committee (Stockholms norra djurförsöksetiska nämnd, Södra Roslags Tingsrätt, Stockholm, Sweden). The study included 18 male Sprague-Dawley rats (BW 250 g), housed under standardized conditions regarding the day/night cycle, temperature and humidity. Anesthesia was induced as a mixture of 3 % isoflurane/air and maintained during surgical procedures at 1.5 %.

Rat brains were evaluated at eight time points, namely 0.5, 1, 3, 6, 12, 24, 48 and 72 h after lesioning, and three animals were added as controls. After two days of beam walking pre-training (Abo *et al.* 2001), a transient focal brain lesion was induced by a compression lesion (Kundrotiené *et al.* 2002) in the right sensory motor cortex. After lesioning, the animals were scored daily in sensory motor ability, using beam walking. Pentobarbital (2.0 ml i.p.) was given as terminal anesthesia; the animals were transcardially perfused, and samples for EM examination were taken from the lesioned area and from contralateral regions corresponding to the lesion. The control animals were subjected to the surgical procedure without compression lesion.

Incomplete ischemia induced by compression

A unilateral transient focal lesion was induced in the right sensory motor cortex by compression (Kundrotiené *et al.* 2002). The animal’s head was fixed in a stereotaxic frame with the tooth bar at –3.3 to +1.5 mm, and the center of the lesion was set to bregma –1.0 mm, 3.5 mm lateral to the midline, according to the brain atlas of Paxinos and Watson. The skull bone over the lesion center was removed, and a compressor piston was placed

at the brain surface. The piston was made of Teflon and had a diameter of 8 mm with the medial part shortened by 2 mm, resulting in a medial-lateral distance of 6 mm. The piston was angled 20 ° from the horizontal plane and slowly lowered 2.8 mm towards the center of the brain; the compression was maintained for 30 min. During the operation body temperature was kept at 37-38.0 °C and constant cardiac and respiratory frequency was maintained.

Behavioral testing

Motor function was evaluated in a beam-walking test with a 7-point rating scale (Feeney *et al.* 1981). The animals were trained for two days before lesioning and tested every day starting at 24 h. The rating was based on the number of foot slips of the hind limb. A score of 7 corresponded to no more than two-foot slips and a score of 1 was given when the animal fell off the beam, thus indicating major impairment. Training and testing sessions were conducted during the morning hours in a quiet room with dim lighting.

Morphology

The animals were transcardially perfused at a pressure of 0.3 bars, initially with 50 ml 0.1 M phosphate buffered saline (PBS) with 500 IU heparin added at pH 7.4, followed by 500 ml 3.0 % glutaraldehyde in 0.1 M PBS at pH 7.4. After perfusion, the brains were dissected and stored overnight at +4 °C in the fixative. Tissue samples were selected according to a standardized procedure supported by a fixture (Fig. 1). The brains were cut in two parts in the lesion center (bregma –0.5 mm), and the caudal cutting face was placed tightly against the fixture wall (Fig. 1b). Coronal slices (~1 mm thick at bregma -0.5 mm to -1.5 mm) were placed on the fixture plate (Fig. 1a) with the brain midline aligned to the central mark. Tissue samples were taken bilaterally, according to the fixture marks, between 2 and 3 mm lateral to midline, and thereafter these two samples were further cut into three specimens, which represented the cortical layers I-II, III-IV and V-IV. Randomized sample orientation during further EM preparation ensured unbiased sampling.

Samples were post-fixed in 1 % osmium tetroxide in 0.1 M PBS for two hours, dehydrated in increasing concentrations of ethanol, embedded in resin Agar 100 and polymerized in blocks at 60 °C. Using an LKB V ultramicrotome, the blocks were cut in semithin sections (1 μm) and the sections were stained with toluidine blue. Cortical layers IV and V were defined and areas of interest selected under LM. Ultrathin sections (~60 nm) were cut from the selected areas, stained with uranyl acetate and lead citrate and examined using two transmission electron microscopes, a Zeiss CEM 900 and a Philips Morgagni 268D.

For semi-quantitative estimation, we developed a standardized evaluation protocol considering quantitative and qualitative parameters. The evaluation was based on the following structural parameters: nuclear condensation, chromatin clumping or margination, cytoplasm condensation and vacuolization, organelle changes and nuclear as well as plasma membrane "blebbing" and rupture. The evaluation also included the counting of different types of cells. Pyramidal cells, interneurons, astrocytes and oligodendrocytes, affected as well as unaffected, were counted separately. The section areas were measured and the cell densities per mm^2 were calculated. Furthermore, the status of the neuropil, considering the profile of dendrites and axons, was qualitatively evaluated. Electron micrographs were made in the areas of interest, and the final evaluation was made from these micrographs.

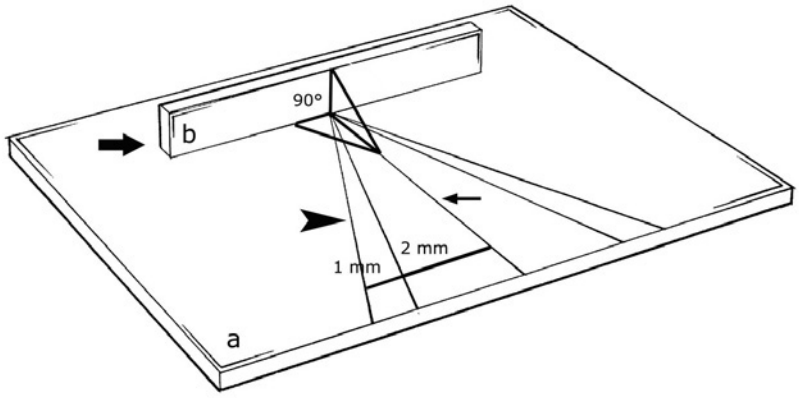

Fig. 1. A fixture made of polystyrene used for standardized sample collection. On the bottom plate (a) a supporting wall (b) is inclined at 90 °. A central mark (thin arrow) is outlined on the bottom plate at an angle of 90 ° angle to the supporting wall. Bilateral help lines (arrowhead) are marked on the bottom plate in order to find the sensory motor cortex at distances of 2 and 3 mm from the central mark.

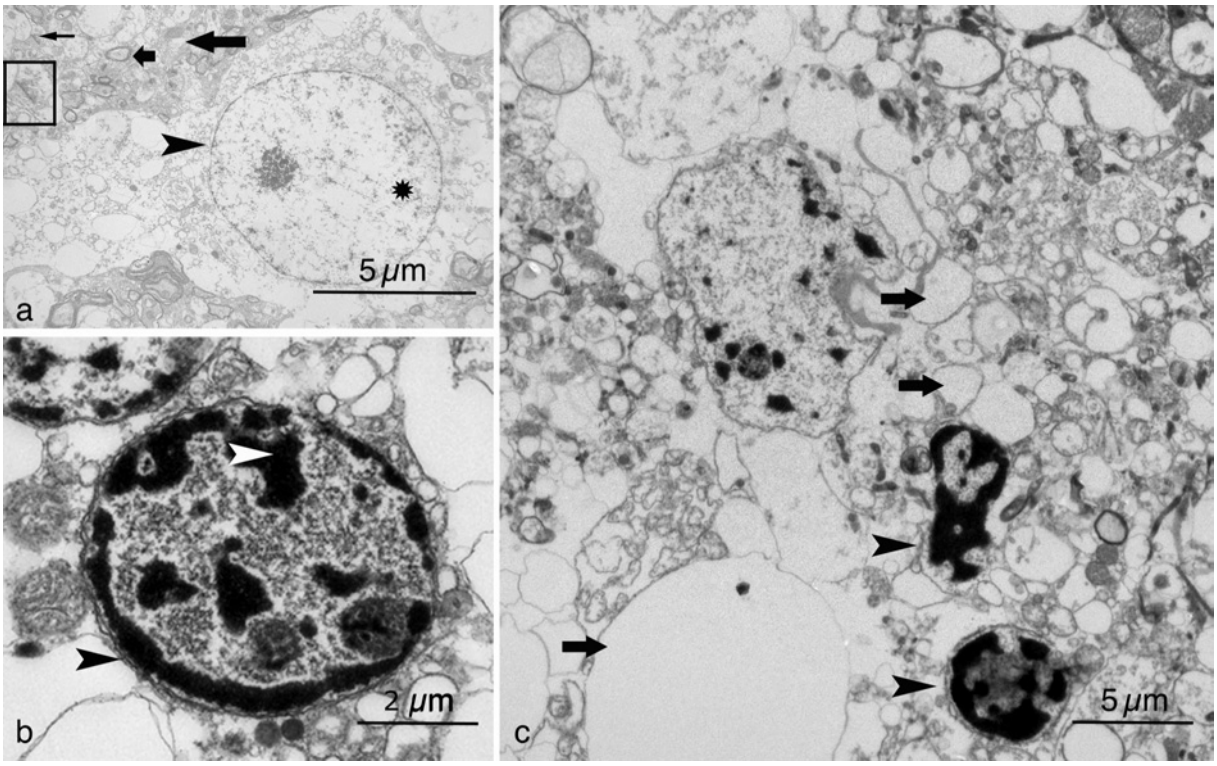

Fig. 2. In control tissue the neuropil (a) showed a normal profile of myelinated axons (block arrow) and dendrites (arrowhead) and axon terminals (thin arrow). The cells in control tissue contained round-shaped nuclei with homogeneously distributed chromatin (asterisk) and intact membranes (arrowheads). An apoptotic cell with highly clumped chromatin (white arrowhead) and intact nuclear membranes (black arrowhead) are shown in (b) and apoptotic bodies (arrowheads) are shown in the vacuolated cytoplasm (block arrow) in (c).

Results

Behavioral test

The animals had the expected functional impairment at 24 h after lesioning, scoring 2 and 3 in the beam-walking test, which indicated a lesion. At 48 and 72 h the beam-walking scores were 5 and 6, respectively, thus indicating functional improvement.

EM evaluation

At the EM level, the neuropil in control tissue was well preserved (Fig. 2a) with a normal profile of myelinated axons, axon terminals and dendrites. As previously described (Cragg 1976), homogeneously distributed nuclear chromatin, distinct mitochondria cristae and distinctly double-lined nuclear and plasma membranes were present (Fig. 2a). A few condensed cells were occasionally noticed.

After evaluating all EM samples in each layer at all post-lesion times, cells with the ultrastructural characteristics of apoptosis, such as clumped chromatin, intact membranes and vacuolated cytoplasm (Fig. 2b, c), were the most prominent cell type. We counted the cells and calculated the cell densities at each time point.

Cell density

In the control tissue cell density was 212.5 cells/mm^2. No marked differences were found in cell densities between the two hemispheres in lesioned brains. At 0.5 h, the total cell density in the lesioned area was 135.2 cells/mm^2 (Fig. 3a), while at 12 h it was decreased to 105.3 cells/mm^2. At 24, 48 and 72 h, the cell densities were nearly the same as in the control tissue, namely 196.4, 186.7 and 191.5 cells/mm^2. At 12 h, the density of apoptotic cells in the lesioned area was 49.9 cells/mm^2, which was 47.3 % of the total cell number. At 24, 48 and 72 h apoptotic cell density was 56.1, 51.8 and 66.8 cells/mm^2, respectively.

At 0.5 h, the total cell density on the contralateral side was 134.5 cells/mm^2 (Fig. 3b); at 12 h the number was decreased to 104.5 cells/mm^2, and at 24, 48 and 72 h, increased densities of 206.1, 203.7 and 205.2 cells/mm^2 were found. Apoptotic cells were at their highest density at 24 and 72 h, when the densities were 21.5 and 21.4 cells/mm^2.

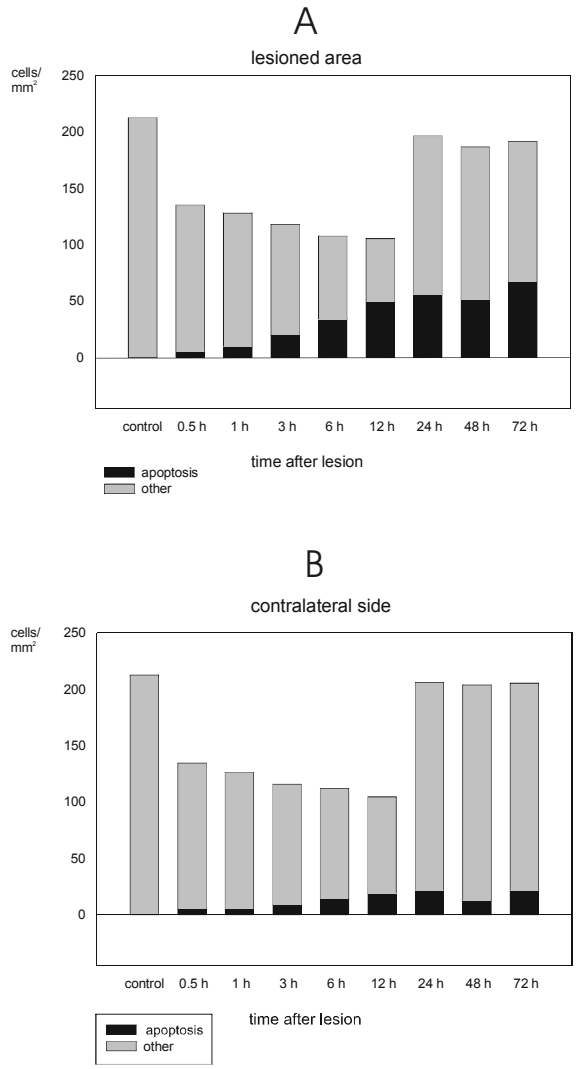

Fig. 3. Stacked column graphs showing the cell density apoptotic cells and the cell density of the remaining cells in the tissue. In control tissue a mixed cell population was found. Panel A shows the cell density in the lesioned area compared to control tissue. Greatly decreased cell density was found at 0.5 - 12 h and the highest density of apoptotic cells at 12 h. Panel B shows the cell density on the contralateral side with decreased cell density at 0.5-12 h.

Ultrastructure

The remaining cells in the tissue were characterized as follows. In the control tissue, cells with intact membranes and homogenously distributed chromatin were observed (Fig. 4a). At all time points in each hemisphere, the tissue in layers IV and V showed a similar ultrastructure. Already at 0.5 h, the neuropil was extended with swollen dendrites and enlarged myelin sheets in the lesioned area (Fig. 4b). At 0.5-1 h slightly shrunken nuclei, cytoplasm vacuoles and expanded ER were observed (Fig. 4b, c), while at 3-6 h the cells were increased in size with enlarged nuclei and diluted cytoplasm (Fig. 4d, e). Chromatin margination was seen at 12 h (Fig. 4f), and cells with disintegrated condensed cytoplasm were found at 24 h (Fig. 4g). Most cells at 48 h contained marked chromatin margination (Fig. 4h), thus indicating an early stage of apoptosis, and some cells also contained cytoplasm vacuolization (Fig. 4h). At 72 h, small bodies with condensed chromatin (Fig. 4f) were found.

On the contralateral side, the neuropil was well organized in all samples with cells containing homogenously distributed chromatin and normal profiles of axons (Fig. 5a). At 0.5 h, a slightly enlarged ER was noticed in the cells (Fig. 5b); the enlargement was more pronounced at 1 h (Fig. 5c). At 3 h large stacks of ER dominated the cytoplasmic compartment in most cells (Fig. 5d). The ER was well organized without internal vacuole formations. At 6-12 h cells were increased in size with enlarged nuclei and slightly heterogeneously distributed chromatin (Fig. 5e, f). Cells with folded nuclear envelopes were present at 24 h (Fig. 5g). At 48 h, some electron-dense cells with condensed nuclei were found (Fig. 5h). These cells showed cytoplasm vacuolization. At 72 h the cells had a normal appearance with homogeneously distributed nuclear chromatin and well-organized cytoplasm (Fig. 5j).

Electron microscopy showed that in the lesioned area the neuropil was extended with swollen dendrites and enlarged myelin sheaths already at 0.5 h post-lesion. Apoptotic cell death was most frequent at 12 h, and only a few necrotic cells were found in the lesion. At 3–24 h, hypertrophic cells were found. On the contralateral side, ER enlargement was already seen at 0.5 h, while at 3 h the ER was present as large stacks in the cytoplasm, indicating secondary delayed cell death.

Discussion

This study demonstrates that a cortical focal compression lesion resulted in apoptotic cell death in the lesioned area, representing a response to transient ischemia and thereby a *penumbra* zone. This is also confirmed by previous studies in which apoptosis was considered to be the main cell death pathway in the *penumbra* zone (Vexler *et al.* 1997, Lee *et al.* 1999, Lipton 1999). In our study apoptosis was most frequent in the lesioned area at 12 h after lesioning, while at 3 h a marked ER enlargement was found on the contralateral side.

The *penumbra* tissue surrounding an infarction is living tissue, the fate of which is undetermined because it could develop into dead tissue. The *penumbra* zone is a

"window" in time and space (Lipton 1999). The changes in this zone are time-limited, time-dependent, and cells might die within hours or days, thus enlarging the necrotic area and moving the zone further from the infarction core. As long as the cells are viable, the *penumbra* zone has the potential to preserve both morphological integrity and functional recovery and is therefore a target for therapy. However, it is known that the *penumbra* zone extends over different areas in different species (Hossmann 1994) and selective cell populations might be vulnerable by subtle changes in blood flow than were examined in this study.

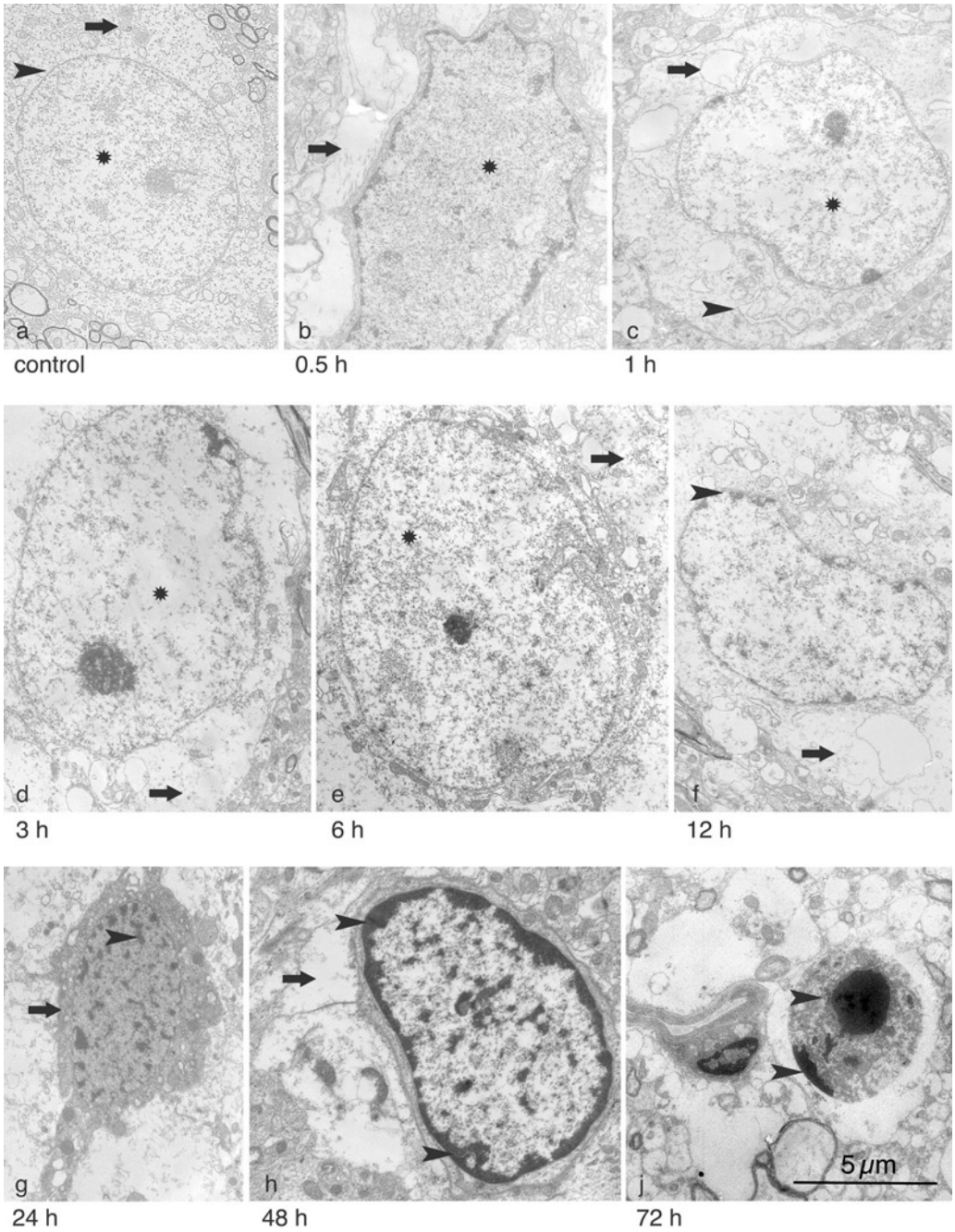

Fig. 4. Electron micrographs showing control tissue and cortical layer V in the lesioned hemisphere. In control tissue (a) cells showed homogeneously distributed chromatin (asterisk), intact nuclear membranes (arrowhead) and distinct organelles (block arrow). At 0.5 h, the neuropil appeared extended with swollen dendrites (b; block arrow). Slightly shrunken nuclei (asterisks) are shown in (b, c) and in (c) expanded ER (arrowhead) and cytoplasmic vacuoles (block arrow). At 3-6 h, enlarged nuclei (d, e; asterisks) were observed and at 3-12 h diluted cytoplasm (d-f; block arrows). Chromatin margination (f; arrowhead) was present at 12 h. Some cells, highly condensed, (g) with disintegrated cytoplasm (block arrow) and large chromatin clumps (arrowhead) were seen at 24 h. At 48 h marked chromatin margination (h; arrowheads) and large cytoplasmic vacuoles (block arrows) were found. Some apoptotic bodies with condensed chromatin (j; arrowheads) were present at 72 h and shown in (j). Scale bar = 5 μm.

Apoptotic cell death is responsible for cell homeostasis in tissues during development (Raff *et al.* 1993). Apoptosis also serves as a defense mechanism to remove unwanted and potentially dangerous cells (Steller 1995). Some authors described apoptosis as a program preferentially triggered by a microcirculatory disturbance (Chopp and Li 1996, Vexler *et al.* 1997). Accordingly, apoptosis would significantly contribute to the neuronal damage in transient ischemia, as was shown in our study.

Apoptosis can be initiated by a number of different stimuli, and many conditions during ischemia offer such stimuli: free radical generation, NO production, reduced mitochondrial membrane potential, increased intracellular calcium levels, activation of calpain and increased expression of p53 protein (Duchen 2000, Sugawara and Chan 2003). Also, a withdrawal of trophic factors, e.g. neurotrophins, has been proposed to cause apoptosis, not only in developing tissue but also during injury (Campenot and MacInnis 2004). The presence of appropriate trophic factors considerably improves the ability of a cell to survive a stressful situation. Therefore, withdrawal of neurotrophins, due to the compression followed by insufficient blood supply, could cause cell damage and subsequently cell death.

In many experiments, apoptotic cells are labeled with markers for DNA fragmentation, e.g. TUNEL staining. As DNA breakdown is considered as the main biochemical criteria for apoptosis, positively labeled cells are considered to be apoptotic. However, DNA fragmentation also occurs in necrosis (Studzinski 1999). Thus, cells positively stained for DNA fragmentation might show characteristics of cell death *per se*, necrotic or apoptotic (Studzinski 1999). Hence, the EM technique is useful in identifying ultrastructural criteria of different types of cell death.

The morphological characteristics of apoptosis are subdivided into three different types of apoptosis (Fawcett *et al.* 2001). The first type has the classical star-shaped appearance with dense pyknotic nuclei and membrane "blebbing". Finally, apoptotic bodies are detached from the cell body. The second type is characterized by the formation of autophagic vacuoles and sometimes the dilation of mitochondria and the endoplasmic reticulum. Some pycnotic nuclei and membrane "blebbing" might be seen. In type three, neither pyknotic nuclei nor membrane "blebbing" are present, but rather a considerable disintegration of mitochondria, the Golgi complex and endoplasmic reticulum. This type of apoptosis is morphologically similar to necrosis.

The apoptotic cells occurring after a compression lesion did not fully fit into any of the three types of apoptosis described above. Nuclear changes, with chromatin aggregation, were strongly associated with apoptosis but the nucleus was not pyknotic. The cytoplasm was highly vacuolated, but no dilation of mitochondria or of the endoplasmic reticulum was found. The cell changes resemble those seen in type two apoptosis, but not to a full extent. It is known that large autophagic vacuoles within the cytoplasm are present in apoptotic cell death during ischemia (Lipton 1999, Fawcett *et al.* 2001). Furthermore, autophagocytotic cell death with a condensed cytoplasm containing large vacuoles and autophagocytomes is described as a separate type of cell death in ischemia (Marzella and Glaumann 1981). This cell death is very similar to apoptotic cell death type two. The damaged cells with irregularly clumped chromatin and cytoplasm vacuoles found in the lesioned area were therefore considered to be apoptotic.

The final stages of apoptosis in ischemic tissue are similar to the end stages of necrosis (MacManus and Linnik 1997), which complicates the differentiation between apoptosis and necrosis. There are also similarities in the ultrastructural criteria of necrosis and apoptosis. Cell membrane "blebbing", swollen mitochondria and cytoplasm condensation combined with vacuolization occur in both forms of cell death (Studzinski 1999). In our study, the characteristics of apoptotic cell death, such as nuclear chromatin margination and clumping, were already found at 0.5 h. These characteristics occurred more frequently over time and reached a maximum at 12 h. Starting at 24 h, some signs of necrosis, such as membrane rupture and vacuolization, were found in the lesioned area, which is supported by earlier results suggesting that apoptosis can progress to necrosis in ischemic tissue.

Since EM pictures represent very selective events in time and space (Lloreta-Trull *et al.* 2000), unbiased selection of the tissue must be ensured. Therefore we developed a standardized sampling procedure supported by a fixture. As the sample orientation was randomized, we considered the final ultrathin sections to be representative for the lesion with respect to both cell number and cell morphology.

Already at 0.5 h after lesioning, energy-requiring apoptotic changes were found in cells in the lesioned area. A reduction of ATP levels during short periods of ischemia has been reported possibly as shortly as 1-2 min

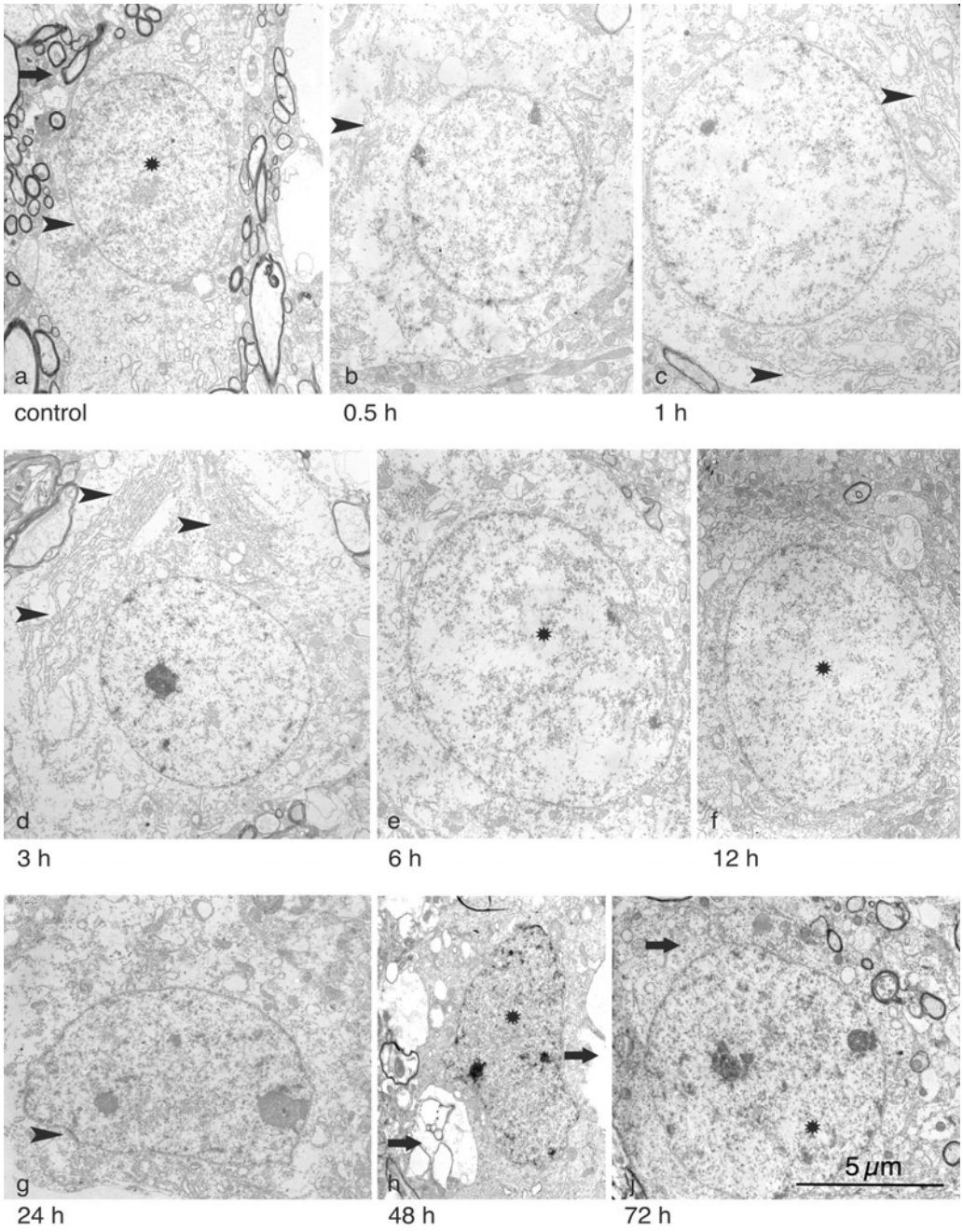

Fig. 5. Electron micrographs showing control tissue and cortical layer V in the contralateral hemisphere. Control tissue (a) shows cells with intact nuclear membranes (arrowhead) and homogenously distributed chromatin (asterisk) and a normal profile of axons (block arrow). Expanded ER (arrowhead) is present in cells at 0.5-1 h in (b, c) and large stacks of ER (arrowheads) dominated the cells at 3 h (d). Enlarged nuclei (asterisks) were present at 6-12 h (e, f) and folded nuclear envelopes (arrowhead) at 24 h (g). At 48 h, (h) cells with condensed chromatin (asterisk) and cytoplasm vacuolization (block arrows) were found. At 72 h (j) the cells showed a normal appearance with homogeneously distributed nuclear chromatin (asterisk). Scale bar = 5 µm.

of ischemia. It has also been speculated that the biomechanical features of different compression lesion models may influence the frequency and regional distribution of apoptotic cell death (Bonfoco *et al.* 1995, Choi 1996) and that the compression of the cortex differs from study to study. Also, the apoptotic process is considered to be a rapid death pathway, as was demonstrated in our study. The number of apoptotic cells was increased at 12 h after lesioning when apoptotic cells were most frequent (47.3 % of the total cell number). In previous time-course studies of apoptotic changes in neurons following controlled cortical impact injury, cells with DNA damage were most apparent 24 h after lesioning. The DNA damage was detected by TUNEL staining, and many of the TUNEL-positive cells failed to exhibit apoptotic morphology. However, the morphology

was examined only by light microscopy and not at the ultrastructural level where subtle changes can be seen.

Massive enlargement of the endoplasmic reticulum is a criterion for secondary delayed cell death and is mostly found in transient global ischemia. As shown previously, secondary delayed cell death is known to develop when ATP levels are 70-80 % of normal. In our study an enlarged ER was found most prominently at 3 h on the contralateral side, following the compression lesion. Information processing mediated by extrasynaptic "volume" transmission, i.e. the movement of neuroactive substances through the volume of the extracellular space (Fuxe and Agnati 1991, Syková 1997, Nicholson and Syková 1998) is probably already damaged at 3 h (Mazel *et al.* 2002) resulting in metabolic disturbances and decreased ATP levels and subsequent apoptotic cell death.

We conclude that the compression lesion is a useful model when studying injured cells. Using electron microscopy, subtle morphological changes can be studied and the criteria of apoptotic and irreversible cell death can be detected. As our study showed, apoptotic cells in the lesioned area, representing 47.3 % of the total cell number at 12 h, we consider the lesioned tissue to be a *penumbra* zone. Since the ultrastructural criteria of secondary delayed cell death were found on the contralateral side at 3 h post-lesion, we suggest 3 h to be a breakpoint at which cells either progress towards lethal changes or recover. Neuronal reorganization could be possible in the *penumbra* zone since metabolically active cells are present.

Acknowledgements

This study was supported by Stiftelsen for Strategisk Forskning (R98:022), Academy of Sciences of the Czech Republic (AV0Z 5039906), Grant Agency of the Czech Republic Contract (304/03/1189).

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