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Apoptosis in the course of experimental intracerebral haemorrhage in the rat

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Intracerebral haematoma was produced in 25 adult rats by infusion of 100 µl of autologous blood into the striatum. The animals' brains were removed at 1, 3, 7, 14 and 21 days after production of the haematoma. The TUNEL method was used to detect DNA fragmentation and TUNEL-positive cells were qualified. TUNEL-positive cells were already found on the first day of observation and were present for three weeks after haematoma production.
These results provide evidence that programmed cell death is associated with intracerebral haemorrhage.

Key words: apoptosis, intracerebral haemorrhage, rats

INTRODUCTION

Spontaneous intracerebral haematoma (ICH) is associated with 50% mortality and result in many survivors suffering neurological disorders [2]. ICH accounts for 14% of all strokes [3, 10]. Understanding the mechanisms of the neuronal injury and functional loss after ICH may facilitate the development of therapeutic strategy. Our previous studies have demonstrated that astroglia and microglia play an important role in the pathophysiological processes in the region of the perihaematoma [8, 9]. Although both necrosis and apoptosis may lead cells to disappear in the course of ICH, the evolution of qualitative and quantitative changes to astroglial and microglial cells in the course of ICH suggest that their elimination may be the result of apoptosis. This process has also been described with regard to other injures of the central nervous system such as traumatic injury or cerebral ischaemia [4, 6]. This study was undertaken to evaluate the occurrence of apoptotic cells in the course of experimental ICH in the rat.

MATERIAL AND METHODS

20 adult rats weighing 275–350 g were used in the study. The animal care and treatment guidelines outlined by the European Community Council Directive of 24th November 1986 (86/609/EEC) and those set by the local committee for ethics were followed. Animals were generally anaesthetised with fentanyl (Fentanyl, Polfa-Warszawa, Poland) at a dose of 0.02 mg/kg and dehydrobenzperidol (Droperidol, G. Richter; Hungary) at a dose of 0.75 mg/kg administered intraperitoneally and midazolam maleate (Dormicum, Roche; Switzerland) at a dose of 0.3 mg/kg administered intramuscularly every 30 minutes. Additionally, the animals received sevoflurane of 2.2 vol% end-tidal concentration immediately after tracheotomy. Sevoflurane (Sevorane, Abbott; United Kingdom) was administered by means of a Sigma Elite Vaporizer (Penlon; United Kingdom). Inspiratory and end-tidal sevoflurane concentrations were monitored with Cardiocap 5 (Datex-Ohmeda, Finland/USA). The animals were divided into five subgroups of four animals per subgroup depending on the length of the survival period: 1, 3, 7, 14 or 21 days.

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Tracheotomy was performed under general anaesthesia with infiltration anaesthesia with 1% lignocaine (Lignocainum hydrochloricum, Polfa-Warszawa; Poland) and an intubation tube was inserted. The animals were mechanically ventilated with the Small Animal Ventilator SAR 830/p (CWE, Inc. USA), using a mixture of air and oxygen (FiO2 = 0.5). Tidal volume and ventilatory frequency were adjusted to keep the end-tidal concentration of CO_2 ($ETCO_2$) constant between 5.2 and 5.5 kPa and O_2 saturation of haemoglobin (SaO_2) above 95%. $ETCO_2$ was monitored with carbon dioxide analyser Capstar 100 (CWE, Inc. USA). Continuous monitoring of the SaO_2 was performed with a pulse oximeter monitor (Novametix; USA) connected to a sensor placed across the animal's hind foot.

Both femoral artery and vein were cannulated for continuous mean arterial blood pressure monitoring, blood sampling and fluid infusion. Body temperature was kept at 37.0°C using a rectal thermometer and a feedback-controlled heating pad (EST, Stoelting; USA).

With the use of an operating microscope a hole was drilled in the frontal bone. After introducing a 26-gauge needle into the striatum (stereotaxic coordinates: B = 1.2 mm; L = 2.5 mm; H = 5.5 mm), 100 μl of autologous non-heparinised blood was injected for 5 minutes, after which the needle was left in place for a further 4 minutes. It was then slowly withdrawn and the skull sealed. Next the rats were allowed to awake under $ETCO_2$ and SaO_2 control. When breathing was spontaneous and values of $ETCO_2$ had fallen below 5.6 kPa and SaO_2 had risen above 95%, we were able to remove the tracheal tube.

Immunohistochemistry

The animals were deeply anaesthetised with lethal doses of thiopental sodium (Thiopental, Biochemie GmbH, Austria) at a dose of 50 mg/kg and then transcardially perfused with a 0.9% solution of NaCl followed by a 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4). The brains were postfixed in 4% paraformaldehyde fixative for 3–4 hours and then kept in 0.1 M phosphate buffer containing 15% sucrose (overnight at 4°C) and 30% sucrose (until sunk). Coronal 10-μm-thick, serial sections of the brain were cut on a JUNG 1800 cryostat (Leica, Germany).

In situ labelling of nuclear DNA fragmentation (TUNEL)

The sections were pretreated with proteinase K (25 μg/ml, Sigma) for 15 min followed by 3% H_2O_2 for 10 min, preincubated in TdT buffer (30 mM Trisma base, 140 mM sodium cacodylate pH 7.2, 1 mM cobalt chloride). The sections were then incubated in a latter buffer containing TdT (0.05 U/μl buffer, Boehringer Mannheim) and biotin-16-dUTP (0.01 nmol/μl buffer, Boehringer Mannheim) in a humid atmosphere and at 37°C for 60 min. The reaction was terminated by washing the slides in 300 mM NaCl, 30 mM sodium citrate for 15 min. Sections were then blocked with 2% BSA (bovine serum albumin; 10 min) and incubated with an avidin-biotin-peroxidase complex (ABC kit, Vectastain, Vector) for 60 min, followed by diaminobenzidine/H_2O_2 and counterstained with cresyl violet. Positive controls included treatment of the sections with DNase. The structures as well as the localisation and morphology of the haematoma were assessed on the sections stained with cresyl violet using Labophot (Nikon, Japan), reference being made to the stereotactic atlas of the rat brain.

RESULTS

Cells with DNA fragmentation were detected by TUNEL staining. TUNEL-positive cells were not observed in the contralateral hemispheres in any of the subgroups of experimental animals. On the first postoperative day a relatively large cavity of haematoma was found. TUNEL-positive cells, probably leucocytes, were present in the clot. In the perihaematoma region a large number of TUNEL-positive cell were observed, which possessed differentiated morphology and staining intensity (Fig. 1A, B; blue arrow). A few apoptotic bodies were found among them (Fig. 1A, B; black arrow). These were characterised by a round shape and strong staining.

Three days after production of the haematoma the morphology of the regions studied was unlike that on the first postoperative day. In the region around the clot both TUNEL-positive cells (Fig. 1C, D; blue arrow) and apoptotic bodies (Fig. 1C, D; black arrow) were noted.

On the 7th day of observation a significant reduction in the morphological diversity of TUNEL-positive cells appeared. TUNEL-positive cells of round shape and strong brown staining predominated in the region enclosed by the haematoma cavity (Fig. 1E, F; black arrow).

After two weeks only single apoptotic bodies were detected in the perihaematoma region (Fig. 1 G, H; black arrow). A marked reduction in the area of the haematoma cavity was observed.

Three weeks after production of ICH the area of the haematoma cavity was covered by a glial scar. In the region of the gliotic tissue numerous typical apoptotic bodies were observed (Fig. 1I, J; black arrow).

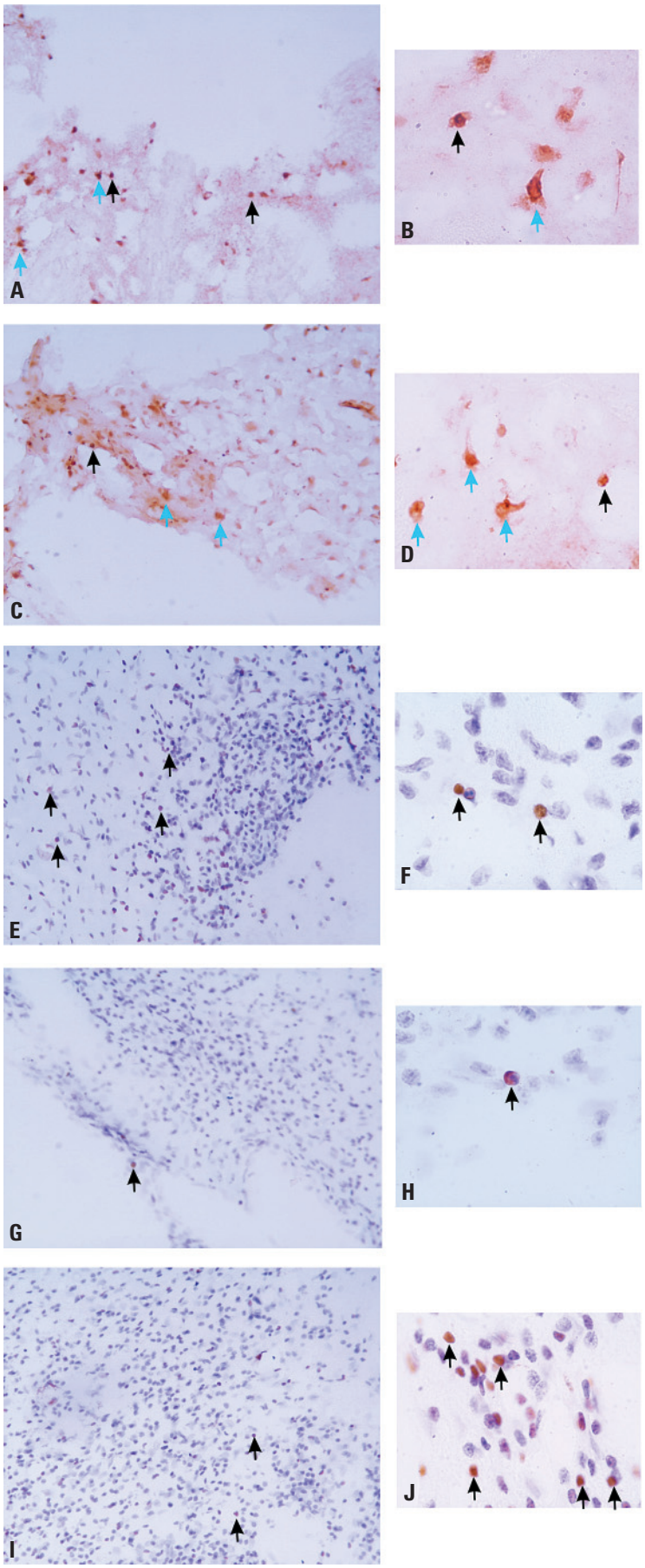

Figure 1. Apoptosis during the period of observation: on the 1st (**A**, **B**), 3rd (**C**, **D**), 7th (**E**, **F**), 14th (**G**, **H**) and 21st postoperative days (**I**, **J**). Black arrows — apoptotic bodies, blue arrows — TUNEL-positive cells. Cresyl violet with TUNEL method. Magnification: 20 × (A, C, E, G, I) and 100 × (B, D, F, H, J).

DISCUSSION

Astroglia and microglia play a key role in the processes of damage and repair of the central nervous system [17]. Signals originated from damaged neurons or induced by changes in the extracellular milieu are likely to be responsible for the activation of astroglia and microglia. The activation of the glial cells may be split into two stages. The first stage (the acute phase) is determined by neuronal degeneration, leucocyte infiltration, the presence of microglia/macrophages and also by structural and metabolic changes in the astrocytes [1]. Activated microglia release several potentially cytotoxic substances such as tumour necrosis factor-alfa (TNF-α) and interleukine 1 beta (IL-1β) [13]. A high concentration of these mediators in the acute phase of ICH plays a key role in subsequent enlargement of the haematoma [16]. Moreover, it is known that TNF-α, and IL-1β are responsible for injury to the blood-brain-barrier, evoking the development of perihaematomal oedema [7]. The present investigation showed that DNA fragmentation occurred in the clot as well as in the region surrounding the clot during the three weeks of observation. In contrast to our findings, Qureshi at al. [15] affirmed that TUNEL-positive cells were present only in the matrix of a haematoma at 24 hours after its production. Differences between experimental models (concerning both protocol and the species investigated) may in part explain the differences in the results. Gong at al. [5] observed TUNEL-positive cells two weeks after the production of ICH. The double labelling study, which showed that most dying cells in the perihaematomal region were neurons whereas only some were astrocytes or endothelial cells, may indicate a greater vulnerability of the former.

At the second stage, which appears in the two weeks following injury, scar tissue is formed [1]. This formation of gliotic tissue, known as the called glial scar, is the result of the repair response of the glial cells. It is composed of the hypertrophied astrocytic processes, macrophages and extracellular matrix [11, 12, 14]. The intense apoptotic process concerning redundant glial cells observed on the 21st day after haematoma production leads to the restoration of the structural and functional integrity of the nervous tissue.

The pathophysiolgy of ICH involves apoptosis both in the clot and in the perihaematoma region. Further investigations are required to define quantitative changes and the involvement of additional factors contributing to neuronal injury in ICH. This may lead to an improvement in the results obtained from therapeutic strategy.

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