ELSEVIER

Developmental Brain Research 116 (1999) 133–140

DEVELOPMENTAL BRAIN RESEARCH

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Research report

DNA damage and DNA damage-inducible protein Gadd45 following ischemia in the P7 neonatal rat

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Accepted 8 June 1999

Abstract

Cerebral ischemia in adult rodents leads to the production of several types of lesions in the genomic DNA, followed by the activation of the damage-response indicator Gadd45. Our purpose was to investigate the structural changes that occur in chromatin DNA and repair processes after ischemic injury in neonatal brain. Neonatal ischemia was induced by the permanent left MCA occlusion in association with 1 h occlusion of the left common carotid artery in 7-day-old Wistar pups. Oligonucleosome fragments that are recognized as the characteristic DNA ladder was observed in a delayed fashion. Double-strand breaks result in high molecular weight fragments of 50- and 300-kbp as demonstrated by pulsed-field gel electrophoresis, and visualized by the TUNEL assay at 24 h of recovery. In contrast, DNA single-strand breaks, shown by the use of DNA polymerase I-mediated biotin-dATP nick translation were not so abundant. Gadd45 immunoreactivity was sequentially increased in vulnerable neurons in the infarct (4 to 24 h) and in sublethally injured neurons in the penumbra (24–48 h). Taken together, these findings suggest that Gadd45 responds to DNA damage following neonatal ischemia. Furthermore, repairing processes seem to be more active in the penumbra and therefore Gadd45 could have also a protective role in cerebral ischemia. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Neonatal ischemia; DNA damage; Gadd45; DNA repair; Apoptosis

1. Introduction

In the first few hours following reperfusion in experimental cerebral ischemia, a burst of highly toxic reactive oxygen species is produced [1]. If oxidative stress is reduced by overexpression of an antioxidant enzyme (e.g., superoxide dismutase) or by gene knockout (e.g., nitric oxide synthase), the volume of damaged brain tissue is reduced [15,17,19,32]. These active oxygen-derived free radicals cause damage to DNA and most of which is repaired a few hours after ischemia [23]. Recently, a group of novel DNA damage- and repair-related genes, termed growth arrest and DNA damage inducible (Gadd) genes, has been identified. These genes were originally isolated in Chinese hamster ovarian cells on the basis of rapid induction par ultraviolet radiation [12]. Among the five Gadd genes, Gadd45 is the only one that has been found to be induced in the brain neurons in vivo by a DNA-damaging agent [38] or by the *N*-methyl-D-aspartate agonist, quinolinic acid [16]. Functionally, Gadd45 protein can stimulate DNA excision repair in vitro by interacting with proliferating cell nuclear antigen (PCNA) [34]. Thus, Gadd45 may be a DNA repair gene. The recent observation that Gadd45 protein is overexpressed in neurons that survive ischemic infarction suggests that this gene may have a protective role in injured brain [6,14,18].

A common cause of brain injury in the perinatal period is hypoxia–ischemia. This kind of injury is thought to be the single largest contributor to static encephalopathies in children and can result in mental impairment, seizures, and permanent motor deficits [35,36]. The traditional model of neonatal hypoxia–ischemia (HI) in a 7-day-old rat was that of Rice–Vannucci which produced neuronal loss in the cortex, hippocampus, striatum and thalamus as well as damage to white matter tracts and gliosis [31]. DNA

Abbreviations: DSBs, double-strand breaks; MCA, middle cerebral artery; PANT, DNA polymerase I-mediated biotin-dATP nick translation; PFGE, pulsed-field gel electrophoresis; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling; SSBs, single-strand breaks

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PII: S0165-3806(99)00084-X

isolated after HI demonstrated internucleosomal fragments in all known areas of damage ipsilateral to the occlusion [11,13,28]. We recently developed a model of transient unilateral focal ischemia in P7 neonatal rats by permanent left MCA occlusion (MCAo) associated with 1-h occlusion of the left common carotid artery. Ischemia with reperfusion in the anastomoses via the carotid artery produced a well-delineated cortical infarct at 48 h of reperfusion and neuronal death with molecular (p53 and Bax increased expression) and morphological (chromatin condensation) features characteristic of apoptosis [29,30]. Furthermore, nitric oxide and peroxynitrite production was reported in such a model, between 24 and 72 h of recirculation [8]. The temporal profile of DNA fragmentation and the potential repair processes after neonatal ischemia, however, have not been explored. This present report adds further weight to the idea that failure of a stress or repair response may be critical in the selective delayed neuronal destruction following neonatal stroke.

2. Materials and methods

2.1. Neonatal ischemia

Experiments involving animals were approved by the French Ethical Science Committee (Statement No. 04223). Ischemia was performed in 7-day-old Wistar rats (15–23 g) of both sexes, as previously described [29]. Rat pups were anesthetized with i.p. injection of chloral hydrate (300 mg/kg). After 15 min, rats were lain on their back and a median incision was made in the neck to expose left common carotid artery. Rats were then placed on the right side and an oblique skin incision was made between the ear and the eye. After excision of the temporal muscle, the cranial bone was removed from the frontal suture to a level below the zygomatic arch. Then, the left middle cerebral artery, exposed just after its apparition over the rhinal fissure, was coagulated at the inferior cerebral vein level. After this procedure, a clip was placed to occlude the left common carotid artery and was removed after 1 h. Carotid blood flow restoration was verified with the aid of a microscope. Both neck and cranial skin incisions were then closed. The surgical procedure lasted about 20 min. During surgery, body temperature was maintained at 37–38°C. Rats pups were placed in an incubator maintained at 37°C until waking up, then transferred to their mother for long time survival.

2.2. Electrophoretic detection of DNA fragmentation

Rats were sacrificed and the brains were rapidly removed, dissected on a cold plate and stored at −80°C.

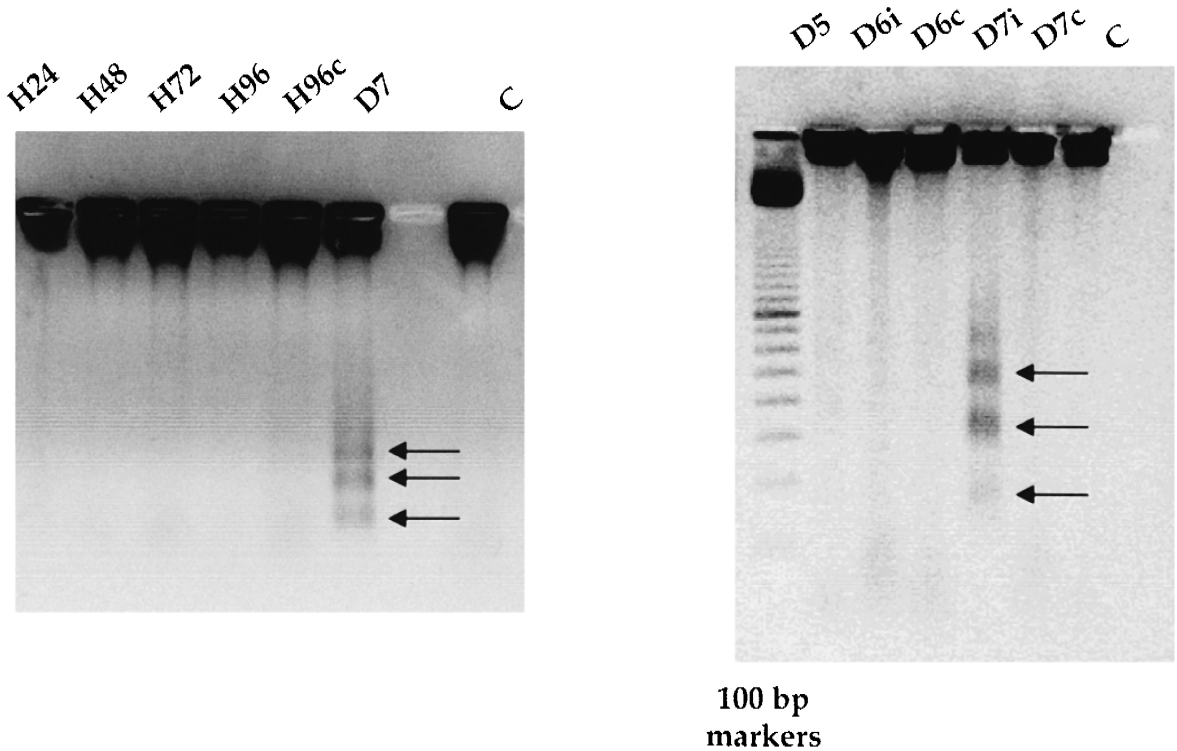

Fig. 1. Typical examples of delayed DNA laddering after ischemia in neonatal rats. At different time points (H, hours; D, days) after left MCAo and 1 h left carotid occlusion, brain tissue was harvested and DNA was isolated from the ipsi- (i) and contra- (c) lateral cortex. DNA fragmentation with formation of ladders was first visualized 7 days (D7) after ischemic insult. No DNA laddering was observed in animals which underwent anesthesia or in naive animals (C), and in contralateral cortex. This experiment was repeat with five other animals with similar results obtained each time.

2.2.1. Conventional agarose gel

Cortical tissues from both lesioned and unlesioned hemispheres were harvested at 18, 24 h and 2, 3, 4, 6 and 7 days after reperfusion ($n = 6$ animals per time point). DNA was isolated as described by Laird et al. [20]. Briefly, tissue was gently homogenized and lysed in 0.5 ml of lysis buffer [100 mM Tris–HCl pH 8.5, 5 mM EDTA, 0.2% sodium dodecyl sulfate (SDS), and 200 mM NaCl)] containing 100 μg/ml proteinase K. After 16 h incubation at 55°C, DNA was precipitated by adding one volume isopropanol with continuous agitation for 15 min at room temperature. Following centrifugation at 13,000 × *g* for 5 min the pellet was air dried and dissolved in TE buffer (several hours) and DNA content determined spectrophotometrically. DNA (10 μg/lane) was electrophoresed on a 1% agarose gel in 100 mM Tris borate (60 V for 4 h) in the presence of 0.3 mg/ml ethidium bromide and visualized with UV illumination.

2.2.2. Pulsed-field gel electrophoresis

Tissues samples, harvested at 6, 9, 18 and 24 h and 2 and 5 days ($n = 3$ animals per time point), were cast in agarose plugs and DNA fragments were separated by pulsed-field electrophoresis as previously described [4]. Electrophoretic conditions and pulse time are indicated in the legend of Fig. 2.

2.3. Tissue preparation

Animals were perfused transcardially with warm heparinized saline followed by a phosphate-buffered solution (0.1 M, pH 7.4) containing 4% paraformaldehyde. Brains were immediately removed and postfixed for 1 h in the same fixative solution at room temperature. Then, they were placed in a phosphate-buffered solution containing 10% sucrose for 2 days at 4°C. The brains were frozen in isopentane at −40°C. They were stored at −80°C until used. Coronal cryostat sections (12 μm thick) were collected on gelatin-coated slides.

2.4. Detection of DNA strand breaks by nick translation

Sections were processed for DNA polymerase I-mediated biotin–dATP nick-translation (PANT) labeling as described by Chen et al. [5]. Briefly, sections were incubated in a moist-air chamber at 37°C for 1 h in the PANT reaction mixture containing 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 20 μg/ml bovine serum albumin, dGTP,

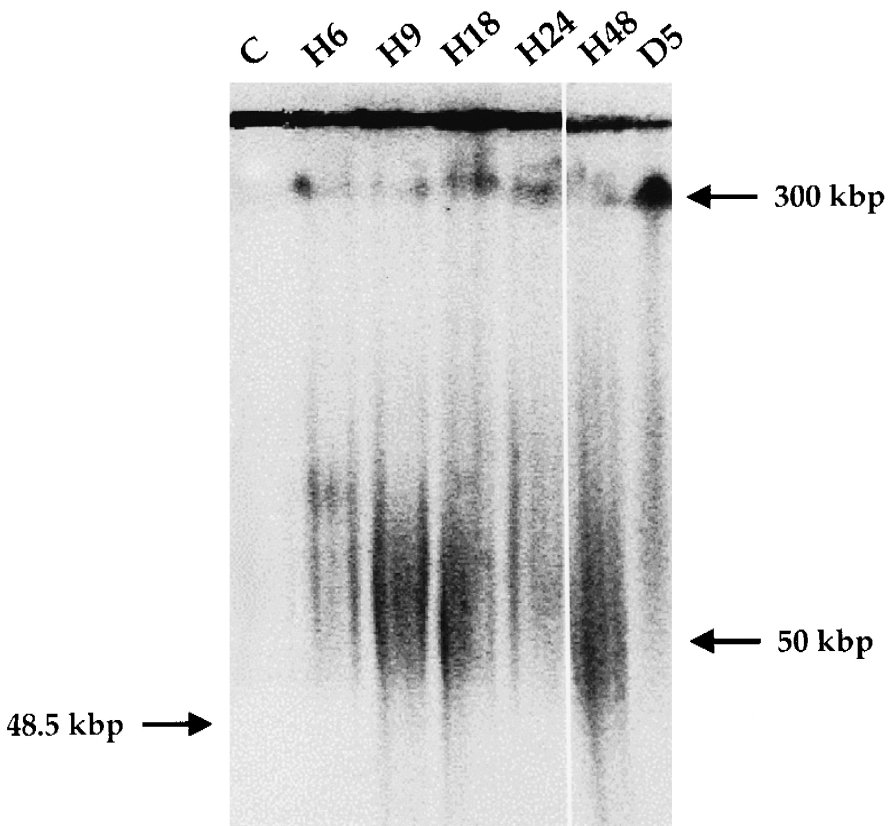

Fig. 2. Pulsed-field gel electrophoresis of DNA plugs from naive (C) and postischemic rat pups at different time points (H, hours; D, days). Unilateral focal ischemia produced the appearance of both 50- and 300-kbp fragments. Note that DNA in wells is lesser at H48 and D5 compared to H24. Molecular weight DNA standards for PFGE were from Gibco BRL (France). Monomer of λ phage is indicated as 48.5 kbp. Electrophoresis was performed at 250 V during 16 h with a 25-s pulse time.

dCTP, and dTTP, at 30 μM each and 29 μM biotinylated dATP, 1 μM dATP and 40 U/ml *Escherichia coli* DNA polymerase I (Sigma) in PBS pH 7.4. After washing, sections were incubated with streptavidin–horseradish peroxidase in PBS (Elite ABC kit, Vectastain, Vector, Biosys, France) and revealed with diaminobenzidine. Negative controls were performed in the reaction buffer without DNA polymerase I.

2.5. Detection of DNA DSBs by nick end-labeling

Sections were processed for TUNEL assay as previously reported [3]. Briefly, sections were incubated with terminal deoxynucleotidyl transferase (0.2 U/μl; Gibco) and biotin–16-dUTP (20 μM; Boehringer Mannhein), then visualized with streptavidin–biotin–peroxidase complex and diaminobenzidine. Positive controls were obtained by pre-treating alternative brain sections with 200 U/ml DNAse I at 37°C for 5 min.

2.6. Immunohistochemical staining of Gadd45

Sections were incubated with the primary antibody to Gadd45 (C-20; used at 1:200; Santa Cruz, Tebu, France) in PBS 0.1 M containing 2% BSA 0.3% Triton-X100 overnight at 4°C. The secondary (anti-rabbit IgG) biotinylated antibodies (1:200–1:400 dilution, Dako, France) were visualized by the avidin–biotin peroxidase (Elite ABC kit, Vector) and diaminodenzidine. Nonspecific peroxidase activity was abrogated by incubating the sections in 2% hydrogen peroxide in 10% methanol at the appropriate stage. As negative controls, alternative sections were incubated in the absence of primary antibody.

2.7. Double staining

To determine if Gadd45 protein is expressed in the same cells that contain DNA strand breaks after ischemia, double staining for Gadd45 immunoreactivity and in situ

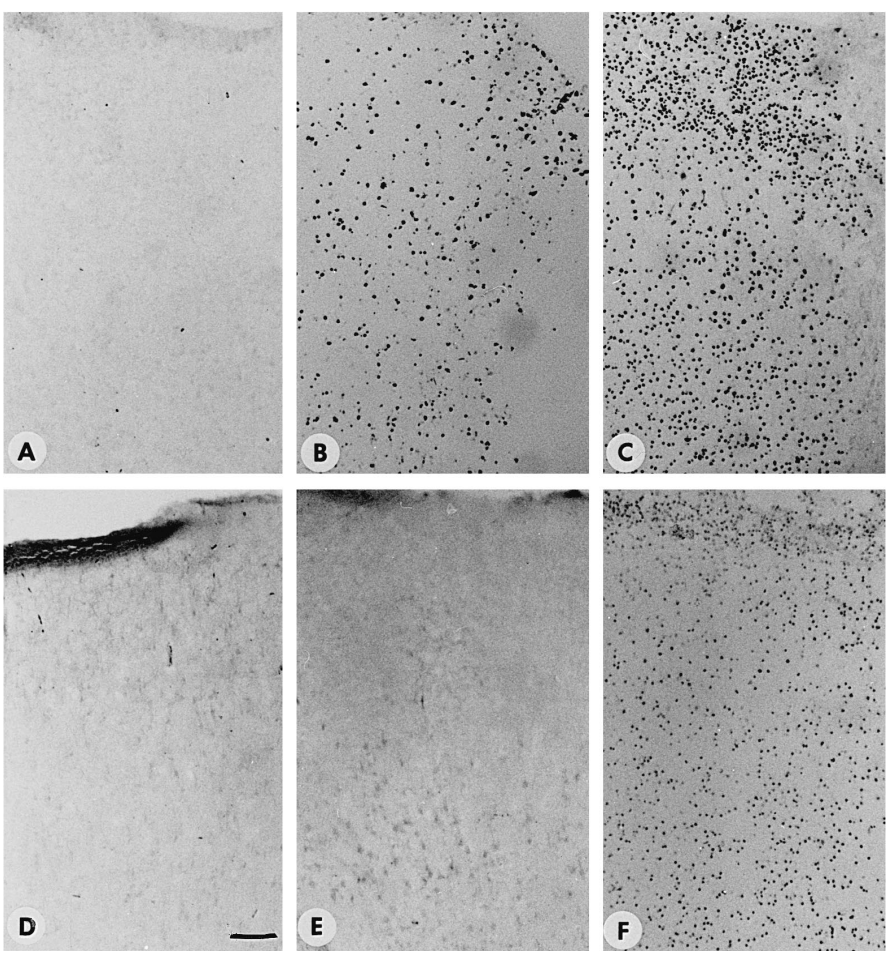

Fig. 3. Representative micrographs of DNA strand breaks in cortical neurons detected by TUNEL assay (A–C) or nick translation (PANT, D–F), followed by 6 (B, E) or 24 h (C, F) of reperfusion. In control (A) a few nuclei demonstrated a TUNEL staining. In contrast, cells exhibited a positive nuclear signal increased from 6 (B) to 24 h (C) of reperfusion. With PANT, staining at 6 h was slightly in deeper cortical layers. This staining increased at 24 h. In both experiments, positive cells had condensed nuclei, and some of them showed nuclear fragmentation or formation of apoptotic bodies at 24 h. Scale bar = 50 μm.

DNA fragmentation was performed in sections obtained at 48 h after ischemia. Brain sections were first processed for Gadd45 immunostaining using the method described above and revealed with DAB enhanced with nickel. The sections

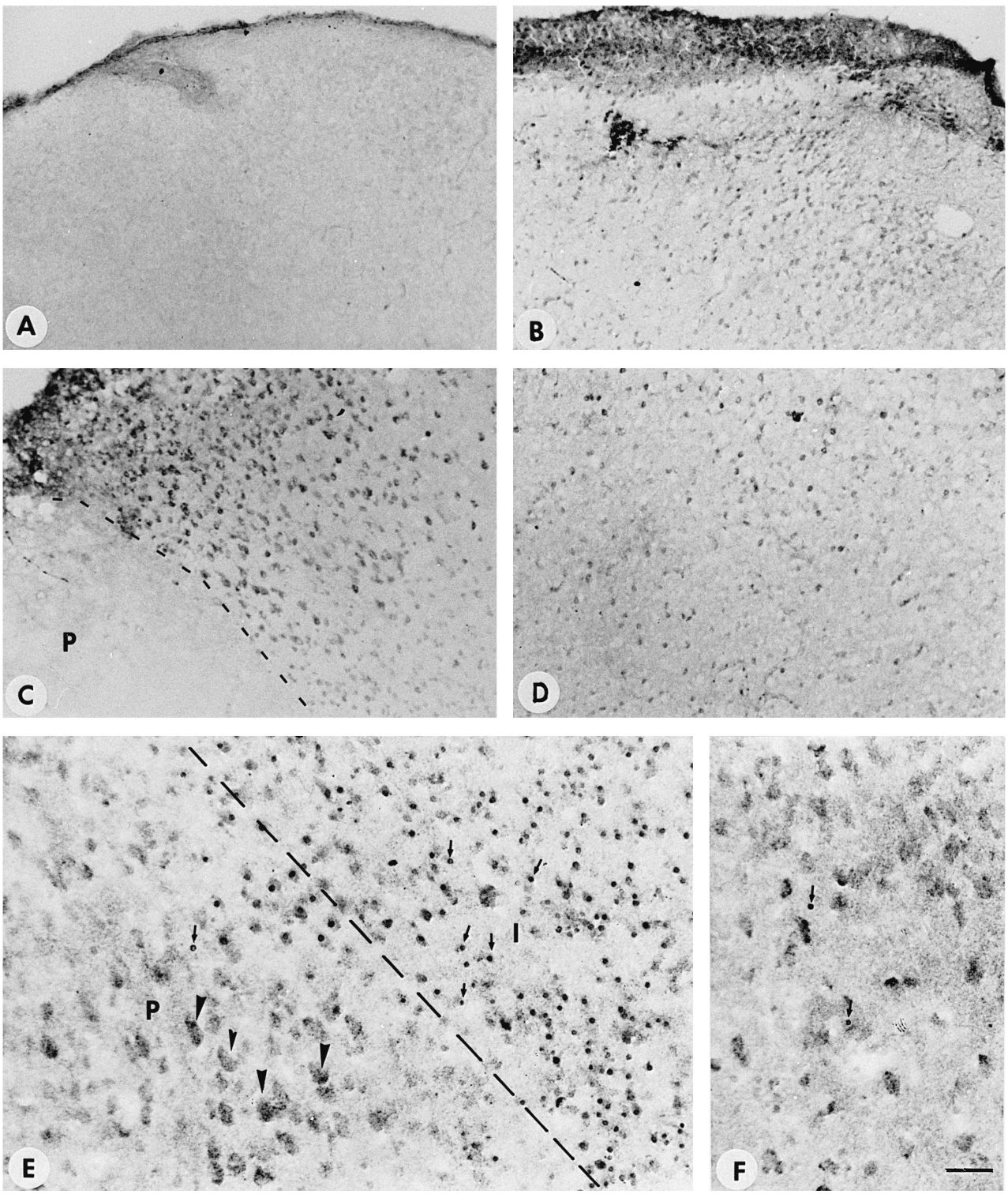

Fig. 4. Representative micrographs of Gadd45 immunoreactivity in contra- (A) and ipsi- (B–F) lateral cortex at 4 (B), 24 (C, D) and 48 h (E, F) after ischemia. Immunoreactivity was increased throughout the ipsilateral cortex at 4 to 24 h. Note that Gadd45 was first detected in layers II and III (B, C; P indicated the penumbra), then in deeper layers (D). (E–F) Double staining experiment (TUNEL assay and Gadd45 immunoreactivity) performed at 48 h of reperfusion. Note the presence of TUNEL-positive nuclei (little arrows at example) in the infarct (I) and Gadd45-positive cells (arrowheads) in the penumbra (P). Only a few TUNEL-positive nuclei could be shown in the penumbra (little arrows in E and F). Scale bar = 50 μm (A–D) and 20 μm (E, F).

were then incubated with TdT and biotin–16-dUTP, and visualized as described above.

To determine if Gadd45 protein is expressed in cell populations other than neurons after ischemia, double immunostaining of Gadd45 and glial fibrillary acidic protein (GFAP, for astrocytes) and of Gadd45 and factor VIII-associated antigen (for endothelial cells and blood vessels) were performed in sections obtained at 48 h after ischemia. In brief, Gadd45 immunostaining was first performed using the procedure described above and revealed with DAB. Then, sections were incubated overnight in buffer containing either anti-GFAP or anti-factor VIII antibodies, followed by an incubation in a biotinylated second antibody and revealed by the avidin–biotin alkaline phosphatase (ABC kit, Vectastain Vector) using Substrate Kit III (Vector™ Blue).

3. Results

3.1. Neonatal ischemia induces DNA fragments and delayed DNA laddering

The bulk of the genomic DNA extracted from naive rat cortex demonstrated that its integrity was roughly preserved, as observed on agarose gels (Fig. 1). The laddering reflective of internucleosomal DNA cleavage was conspicuous in the cortex ipsilateral to MCA and carotid occlusion at 7 days of reperfusion (arrows). On the six brains studied, a ladder was found at 7 days of recovery in five brains, and only one demonstrated a ladder at 6 days postischemia. In the cortex contralateral to the lesioned hemisphere, there was no visible DNA laddering at any time point examined (Fig. 1).

Brain tissue was embedded in agarose, and DNA fragmentation was followed using pulsed-field gel electrophoresis. Under our PFGE conditions, DNA fragments of >1 Mbp were not resolved and DNA extracted from naive pups ($n=3$) essentially remained located in wells (Fig. 2). HMW fragments at 50 and 300 kbp appeared as soon as 6 h postischemia, and increased to 18 and 24 h of reperfusion. Nevertheless, a significant amount of intact DNA remained in wells. In contrast, this amount of intact DNA decreased from 48 h to 5 days postischemia whereas an increase of either 50 or 300 kbp fragments was observed (Fig. 2). In addition, a few 10 kbp fragments could be detected between 4 and 6 days of reperfusion (not shown).

3.2. Neonatal ischemia induces DNA strand breaks

The nick translation method using DNA polymerase I has proved to detect DNA SSBs whereas TdT predominantly catalyses the synthesis of a poly-d-UTP polymer in 3′-OH termini generated by DNA fragmentation [9]. For both PANT and TUNEL, experiments in which DNA polymerase I or TdT was omitted from the reaction mixture resulted in negative staining in all brain sections tested (not shown). In situ DNA fragmentation by the TUNEL assay was found in a few cells within each hemispheric section of naive pups and in the contralateral hemisphere of ischemic brains, which corresponds to the programmed cell death that occurs during development (Fig. 3A). At 6 h of reperfusion, scattered TUNEL-positive nuclei, with chromatin condensation, could be detected in the ipsilateral frontoparietal cortex throughout the cortical layers (Fig. 3B). In contrast, very small numbers of SSB cells were detected by PANT in layers III and IV of parietal cortex (Fig. 3E). PANT-positive cells exhibited necrotic changes, mainly swollen cell bodies or loss of cellular structures. At 24 (and 48) h of reperfusion, the number of both TUNEL- and PANT-positive cells increased in layers II to VI, and light microscopy analysis demonstrated chromatin condensation below nucleus membrane, and/or nucleus fragmentation into rounded or oval bodies (apoptotic bodies) (Fig. 3C, F). Nevertheless, the number of PANT-positive cells was significantly smaller than that of TUNEL-positive cells in all reperfusion times tested.

3.3. Neonatal ischemia induces Gadd45 expression

Low level basal Gadd45 immunoreactivity was seen in brain sections from control rats or in the contralateral hemisphere (Fig. 4A). The expression of this protein increased from 4 (Fig. 4B) to 24 h (Fig. 4C and D) after ischemia in the ipsilateral cortex. Gadd45-positive cells were first shown in the cortical layers II and III (Fig. 4C), then in deeper layers (Fig. 4D). Maximal increases of Gadd-45 positive cells was shown at 48 h in the border of the infarct, in the penumbra (P). To investigate whether the Gadd45 positive cells were damaged, a double staining procedure for Gadd45 and in situ DNA fragmentation by the TUNEL assay was performed. When Gadd45 was highly detectable, Gadd45-positive cells were adjacent to TUNEL-positive nuclei, and cells with TUNEL-positive nuclei were Gadd45 negative (Fig. 4E). In contrast, neurons in the penumbra of the ischemic hemisphere, which were TUNEL negative, appeared to have increased Gadd45 staining (Fig. 4E and F). Double staining was also performed to colocalize Gadd45 immunoreactivity to either astrocytes or endothelial cells and blood vessels. No evidence of increased Gadd45 immunoreactivity in these cell populations was found in any brain sections tested (data not shown).

4. Discussion

There are two main findings in this study. First, DNA DSBs but not SSBs are the primary form of DNA damage in cortical neurons following neonatal ischemia and reperfusion. Second, cellular response to oxidative stress and

DNA damage involve the inducible expression of Gadd45 that enhance defense against oxidative injury or accelerate recovery from cellular injury.

The HMW fragmentation to the 10-, 50- to 300-kbp supports some resemblance to the DNA cleavage demonstrated in cells dying by apoptosis, a process in which the production of 3′-OH end groups predominates, although the responsible endonuclease remains unknown [27,37]. Similarly, such 10, 50 and 300 kbp fragments have been described 18 h following global [14] and focal [4,26] ischemia in adult rats and also following hypoxia–ischemia in P7 rats [14]. Detection of the classical ''DNA ladder'' has been only detected at 6–7 days of reperfusion, whereas such profile was reported as early as 18 to 24 h recovery following focal ischemia in adults [4,24], and 18 h [7,13] or 4 days [28] after neonatal H1. In summary, this wide time window for the development of apoptotic DNA degradation is consistent with previous observation that apoptosis is delayed and progressive after mild transient ischemia in adult rodents [2,10,22]. Furthermore, these results suggested that immature neurons may be more prone to apoptotic death while terminally differentiated neurons exhibit pyknosis or die by necrosis.

The present data showed that DSBs induction was the major event observed following neonatal ischemia. At early and later time points of reperfusion, DBSs were more conspicuously detected than SSBs. These results are in agreement with the wide window of 50 kbp fragments observed and suggested that, in contrast to transient global [23] and focal [5] ischemia in adult rats, oxidative DNA base damage is present but in a lesser extent. In contrast to DS DNA cleavage, DNA nicks should be repaired easily in mammalian cells [33]. Whether a strong DNA repair (by Gadd45 for example) in neonatal brain remains to be determined.

Since cerebral ischemia in rodents is followed by base damage [23] and both SS- and DS-breaks in DNA [4,24–26], it was considered likely that a repair response would be elicited in the injured brain. The data presented here show that Gadd45, is induced in neurons after neonatal ischemia. Such Gadd45 protein upregulation was reported following transient ischemia in adult rats [6,18]. During the first day of reperfusion, expression of Gadd45 is markedly upregulated in neurons located within vulnerable ipsilateral frontoparietal cortex. After longer periods of reperfusion (24 to 48 h after ischemia), increased Gadd45 immunoreactivity is exclusively detected at the periphery of the infarct in the ipsilateral hemisphere. When Gadd45 immunostaining was combined with the TUNEL assay, the protein was absent in frankly damaged cells in the infarct, but was increased in TUNEL-negative cells in the penumbra. These results suggested that: (i) the repair response fails and base damage cannot be replaced (Gadd45 initially present was blocked, maybe by impairment of protein synthesis or degradation by active proteases), and massive DNA fragmentation is occurring; (ii) at later time point, a stress response was detected in potential surviving cells, in which no or very small and undetectable DNA cleavage occurred. The precise mechanism for Gadd45 gene after transient ischemia is currently uncertain. A central role is played by the p53 (wild-type) transcription factor that sits at a crossroad between DNA repair, apoptosis, and cell-cycle arrest [21,39]. DNA damage induces apoptosis, which in turn will initiate a repair response or alternatively involve the cells into an apoptotic pathway of cell death. We recently reported in this model that wt-p53 exerted a significant and time-dependent effect in the initiation of apoptosis, with subsequently Bax protein expression [30]. Therefore, a p53-dependent pathway for Gadd45 expression was observed in vulnerable neurons within the infarct, but also a p53-independent pathway may be present in the sublethally injured neurons of the penumbra.

In conclusion, the results presented here show that one of the DNA repair genes is induced in neurons after focal neonatal ischemia. However, whether the proteins encoded by these genes indeed participate in neuronal DNA repair and consequently have a role in cell survival remains to be determined, namely in neonate.

Acknowledgements

The authors thank I. Jorquera for technical assistance.

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