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Early Decrease in DNA Repair Proteins, Ku70 and Ku86, and Subsequent DNA Fragmentation After Transient Focal Cerebral Ischemia in Mice

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Background and Purpose—Ku70 and Ku86, multifunctional DNA repair proteins, bind to broken DNA ends, including double-strand breaks, and trigger a DNA repair pathway. To investigate the involvement of these proteins in DNA fragmentation after ischemia/reperfusion, Ku protein expression was examined before and after transient focal cerebral ischemia (FCI) in mice.

Methods—Adult male CD-1 mice were subjected to 60 minutes of FCI by intraluminal suture blockade of the middle cerebral artery. Ku protein expression was studied by immunohistochemistry and Western blot analysis. DNA fragmentation was evaluated by gel electrophoresis and terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL). The spatial relationship between Ku expression and DNA fragmentation was examined by double labeling with Ku and TUNEL after reperfusion.

Results—Immunohistochemistry showed constitutive expression of Ku proteins in control brains. The number of Ku-expressing cells was decreased in the entire middle cerebral artery territory as early as 4 hours after reperfusion and remained reduced until 24 hours. Western blot analyses confirmed the significant reduction of these proteins (59.4% and 57.7% reduction in optical density at 4 hours of reperfusion from the normal level of Ku70 and Ku86 bands, respectively; $P<0.001$). DNA gel electrophoresis demonstrated DNA laddering 24 hours after reperfusion, but not at 4 hours. Double staining with Ku and TUNEL showed a concomitant loss of Ku immunoreactivity and TUNEL-positive staining.

Conclusions—These results suggest that the early reduction of Ku proteins and the loss of defense against DNA damage may underlie the mechanism of DNA fragmentation after FCI. **(*Stroke*. 2001;32:1401-1407.)**

Key Words: cerebral ischemia, focal ■ DNA fragmentation ■ DNA repair ■ mice

Cell death linked to oxidative DNA damage has been implicated in the pathogenesis of ischemia/reperfusion injury. The production of reactive oxygen species (ROS) during reperfusion in the ischemic lesion has been known to cause protein oxidation, lipid peroxidation, and DNA damage.[1] Oxidative DNA damage after ischemia/reperfusion was reported to occur in the early stage of reperfusion without cellular injury or DNA fragmentation.[2,3] During this process, the DNA repair enzyme prevents ongoing cell death by repairing damaged DNA, but continuous or severe DNA damage beyond the capability of the DNA repair enzyme triggers a cell-death pathway, such as necrosis or apoptosis, which is dependent on the severity of the insult,[3,4] suggesting that the DNA repair enzyme may have a role in the deterrence of cell-death pathway induction after ischemia/reperfusion. In recent years, DNA damage and repair have been drawing more attention in the field of ischemic neuronal injury; it has been reported that overwhelming DNA damage against DNA repair could induce apoptosis in several different models.[5,6]

Ku70 (70-kDa) and Ku86 (80-kDa) proteins are DNA-binding regulatory subunits of the DNA-dependent protein kinase (DNA PK), which is composed of the 470-kDa catalytic subunit and Ku proteins.[7,8] Ku70 and Ku86 proteins as regulatory parts of the DNA PK initiate the repair process of DNA double-strand breaks, which produce DNA fragmentation, by activating DNA PK after binding to DNA double-strand breaks.[7] In addition to the regulatory function of the Ku proteins in DNA PK, heterodimers of both Ku70 and Ku86 also have independent DNA repair functions, such as single-stranded DNA-dependent ATPase activity and the binding and repair of broken single-stranded DNA, single-stranded nicks, gaps in DNA, and single strand–to–double-strand transitions in DNA.[7,8] The Ku-encoding gene is identical to the human x-ray repair cross-complementing gene group 5 (XRCC5), which is the encoding gene of Ku86, and XRCC6, which is the encoding gene of Ku70.[9,10] A constitutive expression of Ku mRNA has been found in mice.[11]

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Brief ischemia/reperfusion in rabbit spinal cord induces reversible neurological deficits with increased DNA-binding activity of Ku, which is an indicator of activating DNA PK, whereas severe ischemia/reperfusion causes permanent deficits accompanied by decreased DNA-binding activity of Ku, suggesting that Ku is involved in the initiating step of the cell-death pathway, particularly DNA fragmentation in ischemia/reperfusion injury.[12] Recently, we have shown that a decrease in the DNA base excision repair proteins, apurinic/apyrimidinic endonuclease and XRCC1, precedes the occurrence of DNA fragmentation after focal cerebral ischemia (FCI).[13,14] Therefore, we assume that Ku may play a role in cell-death mechanisms, especially DNA fragmentation after cerebral ischemia/reperfusion, and that Ku reduction may involve the mechanism of apoptotic cell death. However, this has not been clearly elucidated.

To explore the change in Ku proteins in the cell-death pathway after cerebral ischemia/reperfusion injury, we examined Ku expression by immunohistochemistry and Western blot before and after FCI and evaluated the temporal and spatial relationship between Ku protein and DNA fragmentation.

Materials and Methods

Focal Cerebral Ischemia

All procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health) and were approved by Stanford's Administrative Panel on Laboratory Animal Care. Male CD-1 mice (35 to 40 g, 3 months old) were subjected to transient FCI by intraluminal middle cerebral artery (MCA) blockade with a nylon suture, as previously described.[15] The mice were anesthetized with 2.0% isoflurane in 30% oxygen and 70% nitrous oxide with the use of a face mask. The rectal temperature was controlled at 37±5°C with a homeothermic blanket. Cannulation of the femoral artery allowed monitoring of blood pressure and arterial blood gases, with samples for analysis taken immediately after cannulation, 10 minutes after occlusion, and 10 minutes after reperfusion (Chiron Diagnostics Ltd). After the midline skin incision, the left external carotid artery was exposed, and its branches were electrocoagulated. An 11.0-mm 5-0 surgical monofilament nylon suture, blunted at the end by heating, was introduced into the left internal carotid artery through the external carotid artery stump. After 60 minutes of MCA occlusion, blood flow was restored by the withdrawal of the nylon suture.

DNA Extraction and Gel Electrophoresis

Animals were killed 4 and 24 hours after ischemia/reperfusion. Ischemic tissue (30 to 50 mg wet weight) was taken from the third 2-mm section along with homologous tissue from the contralateral side after the brain was cut coronally. Samples were incubated overnight in 0.6 mL lysis buffer (0.5% SDS, 10 mmol/L Tris-HCl, and 0.1 mol/L EDTA) with proteinase K (500 μg/mL, Boehringer-Mannheim) at 55°C. The DNA was extracted with equal volumes of phenol and phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated overnight in 0.2 mol/L sodium chloride in 100% ethanol at −20°C. The DNA was washed 2 times with 75% ethanol, air-dried, and resuspended. The DNA concentration was measured with TO-PRO-1 iodide stain (Molecular Probes) and incubated for 30 minutes at 37°C in 0.1 μg/μL DNase-free RNase (Boehringer-Mannheim), and 5 μg per lane was loaded onto an 1.8% agarose gel containing ethidium bromide (0.3 g/mL) and electrophoresed at 100 V for 2 hours. The DNA was visualized and photographed by using UV transillumination (300-nm wavelength).

Detection of Oxidative Cellular Injury After Reperfusion

To confirm the occurrence of oxidative stress, which produces early DNA injury after ischemia/reperfusion, in situ detection of oxidized hydroethidine (HEt) was performed 1 hour and 24 hours after reperfusion by modifying a previously described method.[16] Detection of oxidized HEt is a useful tool to show ROS production because HEt is oxidized to ethidium mainly by the superoxide anion radical.[17,18] Two hundred microliters of HEt (stocked concentration, 100 mg/mL in dimethyl sulfoxide; diluted concentration, 1 mg/mL in PBS with sonication before use; Molecular Probes) was administrated intravenously 5 minutes before the induction of ischemia. The animals were killed 1 hour after reperfusion by transcardial perfusion with 200 mL of 10 U/mL heparin in 0.9% saline and 200 mL of 3.7% formaldehyde in 0.1 mol/L PBS. After postfixation overnight, the brains were sectioned with a vibratome into a thickness of 50 μm at the level of the mid striatum. The sections were then counterstained with Hoechst 33258 (Molecular Probes) for 15 minutes. These sections were observed with a microscope under fluorescent light (HBO, Zeiss). Intensity and expression patterns of the oxidized HEt and nuclear fragmentation as an indication of apoptosis were analyzed after taking photographs with a digital camera (AxioVision, Zeiss) by double exposure to oxidized HEt and Hoechst 33258.

Western Blot Analysis

Whole-cell protein extraction was performed as previously described (n=5 each).[14] Samples were obtained from the ischemic core on the ipsilateral side and from homologous tissue on the contralateral side. Approximately 30 mg of both the ipsilateral and contralateral striata were cut into pieces after 4 and 24 hours of reperfusion and put into 10× vol of Tris-glycine SDS sample buffer (Novex). Samples were then gently homogenized 20 times in a polytetrafluoroethylene Dounce homogenizer (Wheaton). Equal amounts of the samples (10 μL) were loaded per lane. The primary antibodies were either 1:1000 dilution of goat polyclonal antibody against Ku70 and Ku86 (Santa Cruz Biotechnology) or 1:10 000 dilution of anti–β-actin monoclonal antibody (Sigma Chemical Co). For Ku70 and Ku86 detection, Western blots were performed with horseradish peroxidase–conjugated anti-goat IgG with a chemiluminescent kit (Amersham International). As the internal control, Western blot analysis of β-actin was performed with horseradish peroxidase–conjugated anti-mouse IgG reagents (Amersham). After the film was scanned with a GS-700 imaging densitometer (Bio-Rad), a quantitative analysis was performed by use of Multi-Analyst software (Bio-Rad).

Immunohistochemistry of Ku Protein

Anesthetized animals were perfused with 200 mL of 10 U/mL heparin in 0.9% saline and 3.7% formaldehyde in 0.1 mol/L PBS (pH 7.4) after 5 minutes and 2, 4, 8, and 24 hours of reperfusion (n=6 or 7 each), as were normal control animals (n=3). The brains were removed, postfixed overnight in 3.7% formaldehyde, sectioned at 50 μm with a vibratome, and processed for immunohistochemistry. The sections were incubated with blocking solution as previously described[16] and incubated with a primary antibody, goat polyclonal antibody for Ku70 and Ku86, at a dilution of 1:100 (Santa Cruz Biotechnology). The sections were incubated with biotinylated anti-goat IgG (10 μg/mL, Vector Laboratories) and incubated with a complex of avidin and biotinylated horseradish peroxidase (VECTASTAIN Elite ABC Kit, Vector Laboratories). Ku labeling was visualized by using diaminobenzidine (DAB) and nickel chloride as previously described.[16] The nuclei were counterstained with methyl green. As a negative control, sections were incubated without the primary antibody. We also performed the preabsorption method by using Ku-blocking peptides (Santa Cruz Biotechnology) to confirm the specificity of the antibodies.

Double Labeling With Ku Immunohistochemistry and TUNEL

To evaluate the relationship between Ku protein expression and DNA fragmentation, we performed single staining with terminal

deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) and double staining with Ku protein and TUNEL by use of a modified method as previously described.[14] Alternative sections at the level of the caudate putamen were used for Ku70 and Ku86 immunohistochemistry. The sections were immunostained with the Ku70/86 antibody for double staining as described above. Staining was visualized with the use of DAB (0.25 g/L). For single TUNEL staining, the above process was omitted. The slides were incubated for 30 minutes in NeuroPore (Trevigen) for permeation of tissue and placed in 1× TdT buffer (GIBCO-BRL) for 15 minutes, followed by reaction with TdT enzyme (GIBCO-BRL) and biotinylated 16-dUTP (Boehringer-Mannheim) at 37°C for 60 minutes. The slides were then washed twice in 150 mmol/L sodium chloride and 15 mmol/L sodium citrate (pH 7.4) for 15 minutes, followed by a washing in PBS twice for 15 minutes. Avidin-biotin horseradish peroxidase solution (Vector) was applied to the sections for 30 minutes. TUNEL labeling was visualized with DAB (0.25 g/L) and nickel chloride (10 g/L). For single TUNEL staining, the nuclei were counterstained with methyl green.

Quantification and Statistical Analysis

The number of Ku70 and Ku86 immunoreactive cells and methyl green–positive cells was counted in a high-powered field (×400) and expressed as the percentage of Ku-positive cells, as previously described.[14] The number of TUNEL-positive cells was counted as previously described.[9] The statistical comparisons among multiple groups were made by ANOVA, followed by the Fisher protected least significant difference test, whereas comparisons between 2 groups were performed by the unpaired *t* test (StatView, version 5. 01, SAS Institute). Significance between groups was assigned at $P<0.05$.

Results

Physiological Data and Cerebral Infarction

The preischemic physiological values were as follows: mean arterial blood pressure 71.40±3.03 mm Hg, Pa_{O_2} 149.25±20 mm Hg, Pa_{CO_2} 29.05±3.62 mm Hg, and pH 7.34±0.06 (mean±SD). There was no deviation from these values over the period of assessment. An ischemic lesion of the core of the caudate putamen was visible as a pale slightly stained area by cresyl violet staining in the ischemic hemisphere as early as 5 minutes after reperfusion, and it extended to the entire MCA territory at 4 hours by cresyl violet staining (data not shown). The time-dependent increase of infarction in the mouse brains with the use of intraluminal suture blockade is consistent with previous reports that used the same focal stroke model in mice.[9]

DNA Laddering in Gel Electrophoresis of Genomic DNA

To confirm nucleosomal DNA fragmentation, which is characteristic of apoptosis, we analyzed DNA isolated from both the ischemic hemisphere and the contralateral hemisphere. DNA laddering was absent in both the control tissue and ischemic tissue 4 hours after ischemia (Figure 1, lanes 2 and 3). A significant amount of DNA laddering was detected 24 hours after ischemia/reperfusion (Figure 1, lane 4).

Oxidative Cellular Injury After Reperfusion

The production of ROS was shown by oxidized HEt signals as small particles in the cytosol. One hour after reperfusion, the ischemic lesion showed significantly increased oxidized HEt signals (Figure 2A) compared with the nonischemic hemisphere (Figure 2B). Under high magnification, cells with

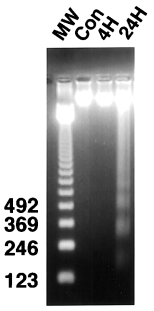

Figure 1. Genomic DNA agarose gel electrophoresis. No DNA laddering was observed in the control brain (Con, lane 2). In the ischemic brain, DNA laddering was evident as bands that were multiples of ≈200 bp 24 hours after ischemia/reperfusion (24H, lane 4) but was not seen 4 hours after reperfusion (4H, lane 3). DNA was labeled with ethidium bromide and electrophoresed on a 1.8% agarose gel. The number of each base pair is shown on the left side. MW indicates molecular weight.

intensive oxidized HEt signals in the cytosol were detected, but cellular injury was not observed 1 hour after reperfusion (Figure 2C), and on the contralateral side, cells with oxidized HEt signals were few, and they were confined to the perinuclear area (Figure 2D). Twenty-four hours after reperfusion, cells with oxidized HEt signals showed nuclear fragmentation, indicative of apoptosis in the ischemic lesion (Figure 2E, arrowheads).

Western Blot Analysis of Ku70 and Ku86 Protein Expression After Reperfusion

Ku70 immunoreactivity was evident as a single band of molecular mass of 70 kDa in the normal brain and contralateral nonischemic brain after reperfusion (Figure 3A, lanes 1 and 3, top). It was decreased 4 hours after reperfusion (Figure 3A, lane 2, top) and was markedly decreased 24 hours after reperfusion in the ischemic lesion (Figure 3A, lane 4, top). Contrarily, a consistent amount of β-actin immunoreactivity is seen at the bottom of Figure 3A, suggesting that the amount of the loaded protein was consistent. Ku86 expression also showed a decrease at 4 hours and a marked decrease at 24 hours (Figure 3B). Statistical analysis confirmed the significant decrease ($P<0.001$) of Ku70 and Ku86 after ischemia (optical density of the Ku70 bands was 2.12±0.22,

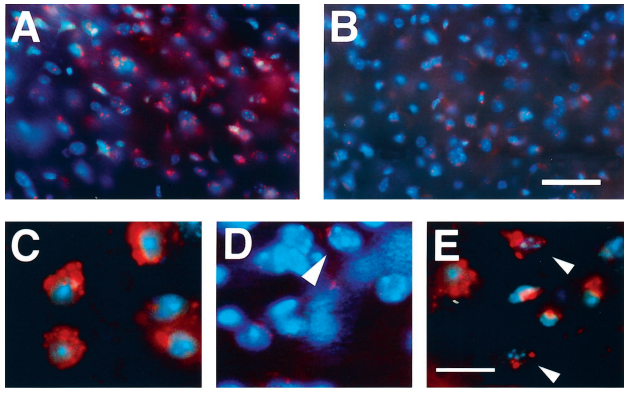

Figure 2. Representative photographs of oxidative cell injury with use of HEt in situ detection after reperfusion. A, Oxidized HEt signals (red) were widely detected in the ischemic lesion 1 hour after reperfusion. B, The contralateral side had fewer oxidized HEt signals. C, Under high magnification, morphologically intact cells were seen with oxidized HEt signals in the cytosol in the ischemic lesion 1 hour after reperfusion. D, In the contralateral hemisphere, few cells with weak oxidized HEt signals around nuclei (blue) were observed (arrowhead). E, Twenty-four hours after reperfusion, nuclear fragmentation with oxidized HEt was observed in the ischemic lesion (arrowheads). Bar=50 μm in panels A and B and 20 μm in panels C through E.

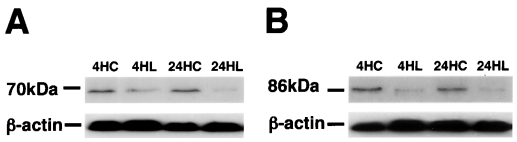

Figure 3. Western blot analysis of Ku70 and Ku86 after ischemia/reperfusion. β-Actin is shown as an internal control (bottom panels). A, Ku70 immunoreactivity was evident as a single band of 70-kDa molecular mass in the whole-cell fraction of the contralateral hemisphere (lanes 1 and 3 [from left to right], top). It decreased 4 hours after reperfusion (lane 2, top) and significantly decreased 24 hours after reperfusion (lane 4, top). The mean optical density of Ku70 in the ischemic lesion was significantly reduced compared with that of the contralateral side, both at 4 hours ($P<0.001$, n=5) and 24 hours ($P<0.001$, n=5) after reperfusion. B, Ku86 immunoreactivity was evident as a single band of 86-kDa molecular mass in the whole-cell fraction of the contralateral hemisphere (lanes 1 and 3, top). It decreased 4 hours after reperfusion (lane 2, top) and significantly decreased 24 hours after reperfusion (lane 4, top). The mean optical density of Ku86 in the ischemic lesion was significantly reduced compared with that on the contralateral side, both at 4 hours ($P<0.001$, n=5) and 24 hours ($P<0.001$, n=5) after reperfusion. H indicates hours; C, control; and L, lesion.

0.86±0.23, 0.28±0.11 and that of the Ku86 bands was 2.15±0.28, 0.91±0.31, 0.25±0.16 in the nonischemic brain, ischemic brain at 4 hours of reperfusion, and ischemic brain at 24 hours of reperfusion, respectively).

Immunohistochemistry and Time Course of Ku Expression After Reperfusion

Immunostaining without a primary antibody or after the preabsorption procedure showed no immunoreactivity (Figure 4A). Ku70 protein was constitutively expressed in the entire region of the normal mouse brain. It was mainly expressed in the nucleus of the cortex (Figure 4B) and caudate putamen (Figure 4C). Ku expression in the contralateral hemisphere after reperfusion was not different from that in the normal brain. Five minutes after ischemia/reperfusion, reduction of Ku70 was observed in the lateral caudate putamen (Figure 4E) but not in the cortex (Figure 4D). After 4 hours of reperfusion, Ku70 expression was reduced in the entire MCA territory cortex (Figure 4F), including the caudate putamen (Figure 4G). This reduction was sustained up to 24 hours in the MCA territory cortex (Figure 4H) as well as in the caudate putamen (Figure 4I). The changes in Ku86 protein expression before and after ischemia/reperfusion were similar to those of Ku70. As shown in Figure 4J and 4K, the number of Ku70- and Ku86-expressing cells was significantly reduced 5 minutes after reperfusion in the caudate putamen ($P<0.05$) and 2 hours after reperfusion in the MCA territory cortex, including the caudate putamen ($P<0.001$). Approximately 70% to 80% of the cells lost their Ku immunoreactivity 4 hours after reperfusion.

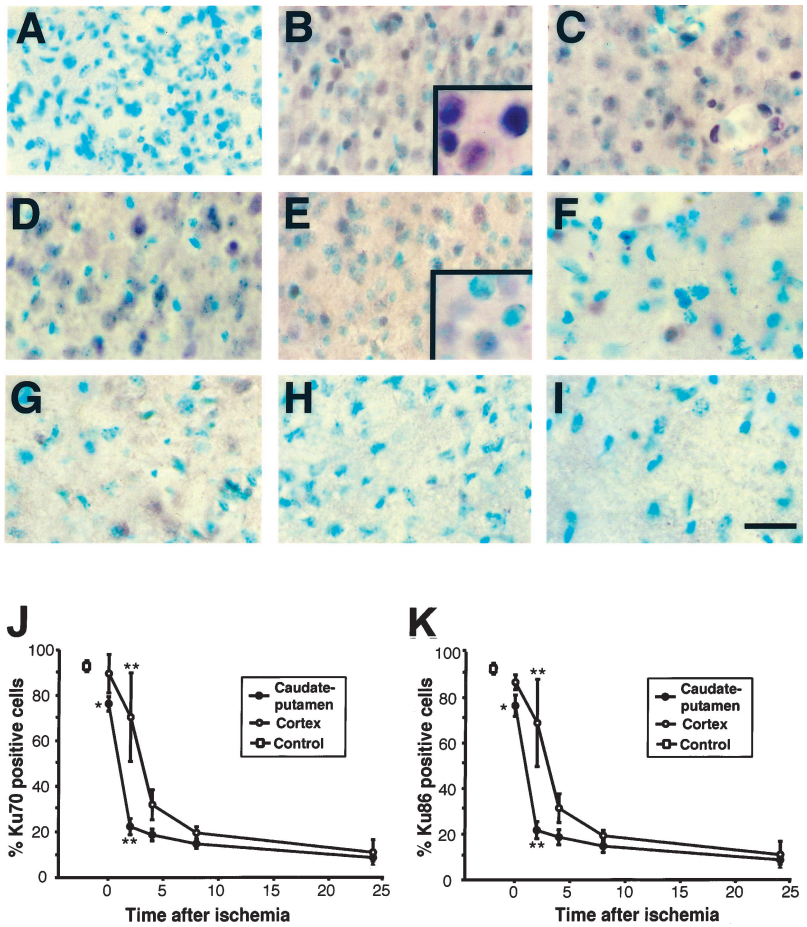

Figure 4. A through I, Representative photomicrographs and the positive-cell count of Ku immunohistochemistry without primary antibody, which showed no Ku immunoreactivity (A). Nuclear distribution of Ku70 was shown in the entire region of the normal brain, including the MCA territory cortex (B) and caudate putamen (C). Five minutes after reperfusion, Ku70 expression was reduced in the lateral caudate putamen (E) but not in the cortex (D). Four hours after reperfusion, Ku70 expression was reduced in the entire MCA territory cortex (F) as well as in the caudate putamen (G). This reduction was sustained 24 hours after reperfusion in both the MCA territory cortex (H) and the caudate putamen (I). Boxes in panels B and E are high magnification of the corresponding area. J and K, Percentage reduction of Ku-positive cells after reperfusion (Ku70 in panel J, Ku86 in panel K). A significant reduction was seen in the caudate putamen as early as 5 minutes after reperfusion, whereas no significant difference was observed in the MCA territory cortex. Two hours after reperfusion, a significant Ku reduction was also observed in the MCA territory cortex. Data represent mean±SD (n=6 to 7). Asterisks indicate a significant decrease in the percentage of Ku-positive cells compared with the normal control cells (*$P<0.05$ and **$P<0.001$, Fisher's protected least-significant difference test). Bar=30 μm in panels A through I.

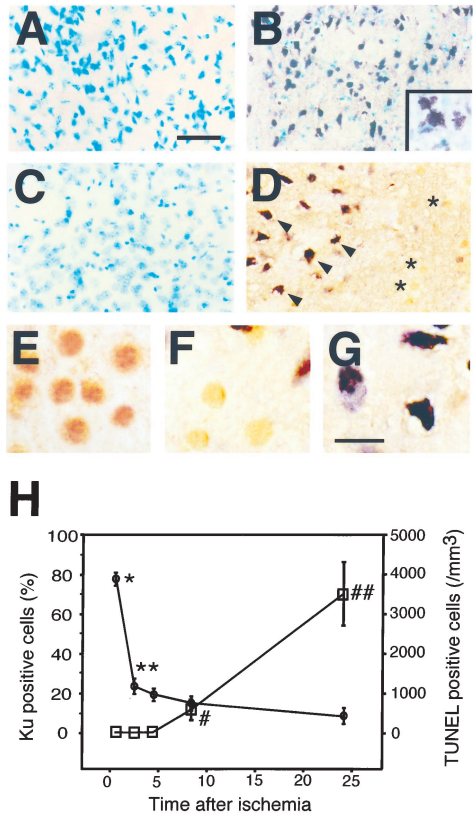

Figure 5. Spatial and temporal relationship between Ku immunoreactivity and TUNEL after ischemia/reperfusion. A through C, TUNEL. No TUNEL-positive cells were seen in the caudate putamen 4 hours after reperfusion (A). TUNEL-positive cells were widely distributed in the caudate putamen 24 hours after reperfusion (B). Highly magnified photomicrographs showed fragmented TUNEL in the nuclei that resembled apoptotic bodies (B, box). TUNEL-positive cells were not detected on the contralateral side (C). D through G, Double staining with Ku and TUNEL. Significant amount of densely labeled TUNEL-positive cells were observed 24 hours after reperfusion (D, arrowheads), whereas some cells showed a faint expression of Ku without TUNEL labeling (D, stars). Under high magnification, cells in the nonischemic caudate putamen showed prominent nuclear Ku expression (E). Ku-expressing cells had no TUNEL (F). TUNEL-positive cells showed no Ku immunoreactivity (G). H, Temporal profiles of the percentage of Ku-positive cells and TUNEL-positive cells in the caudate putamen after transient FCI. Ku-positive cells (%) are expressed as the average of Ku70- and Ku86-positive cells (%). *$P<0.05$ and **$P<0.001$ compared with control brain; #$P<0.05$ and ##$P<0.001$ compared with control brain. Bar=30 μm in panels A through D and 20 μm in panels E through G.

Relationship Between DNA Fragmentation and Reduction of Ku Expression

No TUNEL-positive cells were seen in the ipsilateral caudate putamen 4 hours after reperfusion (Figure 5A). TUNEL-positive cells were widely distributed 24 hours after reperfusion, showing apoptotic characteristics such as condensed or fragmented nuclei with small particles in the nuclei that resembled apoptotic bodies (Figure 5B). No TUNEL-positive cells were detected on the contralateral side (Figure 5C). Double staining with Ku and TUNEL 24 hours after reperfusion showed that most TUNEL-positive cells did not show Ku immunoreactivity (Figure 5D, arrowheads). In contrast, Ku-immunoreactive cells were not TUNEL positive (Figure 5D, stars). At a higher magnification, cells in the contralateral nonischemic brain showed much stronger nuclear Ku immunoreactivity (Figure 5E) compared with the TUNEL-negative cells in the ischemic lesion (Figure 5F). TUNEL-positive cells in the ischemic brain showed condensed and fragmented nuclei without Ku immunoreactivity (Figure 5G). Temporal profiles of DNA fragmentation and Ku expression showed that a reduction in Ku-positive cells preceded an appearance of TUNEL-positive cells (Figure 5H).

Discussion

The present study shows that early reduction in Ku, a DNA repair enzyme, may underlie, in part, the mechanism of cell demise through the oxidative stress–DNA damage–DNA fragmentation pathway after FCI. This conclusion is based on our findings that a marked DNA fragmentation by genomic DNA analysis (Figure 1), which was temporally accompanied by obvious ROS production in apoptotic cells with fragmented nuclei confirmed by HEt in situ and Hoechst nuclear staining (Figure 2), was observed in the ischemic lesion 24 hours, but not 4 hours, after reperfusion, whereas Ku expression, which was detected constitutively in the normal mouse brain, was significantly reduced in the ischemic lesion as early as 4 hours after reperfusion, and the reduction of Ku remained reduced until 24 hours after reperfusion, determined by Western blot (Figure 3) and immunohistochemistry (Figure 4). Furthermore, temporal and anatomic relationships between the loss of Ku expression and DNA fragmentation were shown by double staining with Ku and TUNEL (Figure 5).

This is a first demonstration of the close relationship between Ku reduction and DNA fragmentation in mice after cerebral ischemia/reperfusion. Ku is known to play a critical role in the repair of DNA double-strand breaks by activating DNA PK as a regulatory component; moreover, Ku itself stabilizes broken DNA ends, brings them together, and prepares them for ligation and removal.[7,19] On the basis of these findings, it is conceivable that Ku proteins may play a role in the mechanism of DNA fragmentation, which is destined to cause apoptosis, after FCI. However, little is known about the interaction between Ku and DNA-fragmented cell death after FCI. In the present study, the expression of Ku in Western blot analysis was decreased after ischemia/reperfusion, whereas the expression of β-actin was without effect (Figure 3). These data suggest that the reduction of Ku was not caused by nonselective degradation of proteins after ischemia. Moreover, double labeling with Ku and TUNEL staining revealed that all TUNEL-positive cells had completely lost Ku immunoreactivity. In addition, the quantitative analysis of TUNEL-positive and Ku-immunopositive cells clarified that the decrease in Ku-immunopositive cells preceded the increase in TUNEL-positive cells (Figure 5). These data suggest that the loss of Ku and the failure of the DNA repair mechanism might contribute to DNA fragmentation after FCI in mice. Although there are contradictory reports indicating that a change in Ku was not observed[20] or that Ku was significantly decreased in the cells undergoing apoptosis by potent apoptotic stimuli,[21] a recent study has shown that Ku-deficient cells in mutant mice are extremely sensitive to apoptotic stimuli and show

more DNA fragmentation than do cells in wild-type mice because of the deficiency in DNA double-strand break repair.[22] These data performed in mutant mice with a Ku deficiency agree with the present proposal that the loss of Ku may contribute to the mechanism of DNA-fragmented cell death after FCI.

However, the exact mechanism by which the selective reduction of Ku occurs after FCI is unclear. It has been reported that caspase-3 is responsible for the proteolysis of the 470-kDa catalytic subunit of DNA PK, a catalytic component in the DNA PK complex,[23] and that reduction of Ku protein levels and binding activity in the cells undergoing apoptosis were blocked by treatment with a caspase-3 inhibitor.[16] These reports suggest that the caspase cascade during apoptosis may involve the selective degradation of Ku. Furthermore, using ischemia/reperfusion in rabbit spinal cord, a recent study demonstrated the reduction of Ku DNA binding activity (which is critical to DNA PK activation) after prolonged ischemia that was accompanied by a reduction in the Ku protein and the proteolysis of poly(ADP-ribose) polymerase, one substrate of caspases.[12] This suggests that the reduction of Ku might be involved downstream from the caspase-activating apoptotic pathway after ischemia/reperfusion injury. However, other possible causes of Ku reduction, such as a decrease in Ku protein synthesis, including translational dysfunction or a change in posttranslational regulation after ischemia/reperfusion, remain to be determined.

The present study demonstrated that oxidized HEt signals, indicative of oxidative stress, were increased in the early ischemic lesion without morphological cellular changes as early as 1 hour after reperfusion and that the nuclear fragmented cells with oxidized HEt signals were detected 24 hours after reperfusion (Figure 2). This may suggest that early and sustained oxidative stress is involved in the relatively delayed apoptotic DNA fragmentation after ischemia/reperfusion. Furthermore, the most rapid decline in the early reduction of Ku occurred at 30 minutes to 2 hours in the caudate putamen and at 2 to 4 hours in the MCA territory cortex by the quantitative analysis of Ku immunohistochemistry (Figure 4), suggesting that the full-scale loss of Ku may occur in the ischemic lesion after a short time frame after reperfusion. It has been reported that oxidative stress and reversible DNA damage, which is recovered by DNA repair enzymes, occur immediately after reperfusion in the ischemic lesion and that irreversible DNA damage follows sustained oxidative DNA injury that exceeds the controls of the DNA repair system.[2,3] Thus, the present data suggest that the reduction in Ku and the subsequent failure of DNA repair may be linked with the mechanism of irreversible oxidative DNA injury. Although the cause of Ku reduction is unclear, using mutant mice that overexpress copper/zinc superoxide dismutase, we have recently shown that the copper/zinc superoxide dismutase cytosolic antioxidant attenuates the reduction in apurinic/apyrimidinic endonuclease, another DNA repair protein, and the subsequent DNA fragmentation after FCI[24] supports this idea. However, it is still not known whether ROS degrades the Ku protein directly or secondarily through another oxidative stress–triggered process, such as caspase activation. Nevertheless, ROS may directly contribute, in part, to the reduction in the Ku protein after FCI, inasmuch as ROS could reduce DNA repair activity[25] and directly damage the DNA repair enzyme.[26] In the case of severe oxidative DNA damage against DNA repair, the activated p53 protein has been shown to induce apoptosis directly or through the activation of other apoptosis-regulating genes, such as bax.[27,28] Moreover, the finding that the reduction in p53 prevented apoptotic cell death induced by a deficiency in XRCC4, one of the DNA repair proteins that responds to x-ray–sensitive DNA damage, in mutant mice[29] suggests that p53 may be involved in the apoptosis cascade triggered by a defective DNA repair mechanism. As another subcellular mechanism of apoptosis induced by oxidative DNA damage, it has been proposed that overactivation of poly(ADP-ribose) polymerase by severely damaged DNA may trigger apoptosis through excessive energy consumption during the DNA repair process.[30,31]

In conclusion, we have shown that Ku proteins were rapidly decreased as early as 4 hours after ischemia/reperfusion and that DNA-fragmented cell death occurred after the reduction of Ku expression. These results suggest that the loss of Ku proteins may produce incomplete DNA repair and might be responsible for subsequent DNA fragmentation after transient FCI.

Acknowledgments

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