Distinct Time Courses of Secondary Brain Damage in the Hippocampus Following Brain Concussion and Contusion in Rats

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Secondary brain damage (SBD) is caused by apoptosis after traumatic brain injury that is classified into concussion and contusion. Brain concussion is temporary unconsciousness or confusion caused by a blow on the head without pathological changes, and contusion is a brain injury with hemorrhage and broad extravasations. In this study, we investigated the time-dependent changes of apoptosis in hippocampus after brain concussion and contusion using rat models. We generated the concussion by dropping a plumb on the dura from a height of 3.5 cm and the contusion by cauterizing the cerebral cortex. SBD was evaluated in the hippocampus by histopathological analyses and measuring caspase-3 activity that induces apoptotic neuronal cell death. The frequency of abnormal neuronal cells with vacuolation or nuclear condensation, or those with DNA fragmentation was remarkably increased at 1 hr after concussion (about 30% for each abnormality) from the pre-injury level (0%) and reached the highest level (about 50% for each) by 48 hrs, whereas the frequency of abnormal neuronal cells was increased at 1 hr after contusion (about 10%) and reached the highest level (about 40%) by 48 hrs. In parallel, caspase-3 activity was increased sevenfold in the hippocampus at 1 hr after concussion and returned to the pre-injury level by 48 hrs, whereas after contusion, caspase-3 activity was continuously increased to the highest level at 48 hrs (fivefold). Thus, anti-apoptotic-cell-death treatment to prevent SBD must be performed by 1 hr after concussion and at latest by 48 hrs after contusion.

Keywords: secondary brain damage; apoptosis; brain concussion; brain contusion; rat

Posttraumatic delayed cell damage following traumatic brain injury (TBI) is known as secondary brain damage (SBD) (Cherian et al. 1996). SBD is caused in the cornu ammonis (CA) region of hippocampus by apoptosis (Rink et al. 1995; Vitarvo et al. 2004; Ynag and Xue 2004) that is triggered by caspase-3 (Earnshaw 1995; Wolf et al. 1999). SBD, which is caused at 1-72 hrs after initial brain injury, often induces brain functional disorder (Basset and Slater 1990; Jiang et al. 2000). At present, despite a use of anti-apoptotic-cell-death treatments, it is still difficult to prevent the SBD following brain injury (Uchida et al. 2008). A reason of this difficulty is because the time course of SBD and the appropriate therapeutic period for SBD are different depending on a pathological state of the brain injury, which can be classified into concussion and contusion (Kaijser 1955). But the differences in the time course of SBD between them were not clarified.

In this study, in order to establish an appropriate therapeutic period for treating SBD, we compared the time courses of the posttraumatic apoptotic delayed cell death between post-concussion and post-contusion brains using rat models, by focusing on the histopathological changes in the CA region. To elucidate the localization and the frequency of apoptotic delayed cell death, we also analyzed caspase-3 activity in the CA region and cerebral cortex.

Materials and Methods

All experiments in this study were carried out in accordance with the guidelines for animal experiments issued by the Nihon University College of Bioresource Science.

Experimental model rat groups

Seventy-six male Fisher rats (10 weeks old, 210~290 g body weight) were used in this study. They were purchased from a commercial breeder (Japan SLC Co., Ltd., Tokyo, Japan), and three or four rats were housed in a plastic cage and fed commercial pellets (solid food for laboratory animals, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum. We divided the rats randomly into 3

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groups according to the method to induce traumatic brain damage: concussion model group, contusion model group, and pre-injury group. We subdivided both the concussion and the contusion model group rats into 3 groups depending on the timing of their sacrifice and brain removal: the 1 hr group (the brain was removed at 1 hr after the initial injury), the 48 hrs group, and the 72 hrs group. Accordingly, we divided the rats randomly into the following 7 groups:

Concussion model group: 1 hr group \( (n = 11) \), 48 hrs group \( (n = 11) \), and 72 hrs group \( (n = 11) \).

Contusion model group: 1 hr group \( (n = 11) \), 48 hrs group \( (n = 12) \), and 72 hrs group \( (n = 10) \).

Pre-injury group \( (n = 10) \): Tissue samples were obtained immediately after craniotomy, and no traumatic brain injury was induced.

Induction of brain concussion and contusion

The concussion and contusion injuries were produced in the right parietal cortex using modified versions of the Kuwabara (2002) and Sergienko models (1990). We fixed the rats in the prone position on a stereotaxic frame (SR-5N 96007, Narishige factory, Tokyo, Japan) and anesthetized them using an intraperitoneal injection of sodium pentobarbital (40 mg/kg) (Nembutal, Dainippon Pharmaceutical, Osaka, Japan). Then, we shaved and cleaned their scalps with 70% isopropyl alcohol. After making a midline scalp incision, we reflected the right temporal muscles and carried out a craniectomy of 7 mm in diameter with a dental drill (Viva-mate Plus, Nakanishi Co., Ltd., Tokyo, Japan) and a 1.2-mm bit on the right parietal bone of all rats under surgical loupe visualization (SurgeTel EVC250, KYSMAQ Co., Ltd, Tokyo, Japan). We kept the dura mater intact during this procedure. We induced concussion via a stainless steel rod (weight: 11 g; diameter: 0.5 cm; length: 6.5 cm) with a rounded surface. The injury apparatus was a 10 cm guide tube, which had a 6-mm inner diameter and holes that allowed air to escape without any increase in pressure. The apparatus was positioned perpendicular to the burr hole, and we dropped the weight vertically through the tube from a height of 3.5 cm onto the exposed dura. On the other hand, we induced contusion by electrically burning the cerebral cortex. After craniectomy in the manner described above, we inserted an iron wire-loop electrode with a diameter of 2 mm into the cerebral cortex through the exposed dura. We induced a 5-watt high-frequency current through the iron wire-loop electrode for 5 sec using an electric cautery (Hi-na dial termoMB122 bipolar scissors set, Kirikanyoukou Co., Ltd., Tokyo, Japan). This electrical burn did not invade the CA region. After the induction of brain concussion and contusion, we sutured the line of the scalp incision with silk braid (sterilized braid silk 4/0, Akiyama factory, Tokyo, Japan). Then, we shaved and cleaned their scalps with 70% isopropyl alcohol. After making a midline scalp incision, we reflected the right temporal muscles and carried out a craniectomy of 7 mm in diameter with a dental drill (Viva-mate Plus, Nakanishi Co., Ltd., Tokyo, Japan) and a 1.2-mm bit on the right parietal bone of all rats under surgical loupe visualization (SurgeTel EVC250, KYSMAQ Co., Ltd, Tokyo, Japan). We kept the dura mater intact during this procedure. We induced concussion via a stainless steel rod (weight: 11 g; diameter: 0.5 cm; length: 6.5 cm) with a rounded surface. The injury apparatus was a 10 cm guide tube, which had a 6-mm inner diameter and holes that allowed air to escape without any increase in pressure. The apparatus was positioned perpendicular to the burr hole, and we dropped the weight vertically through the tube from a height of 3.5 cm onto the exposed dura. On the other hand, we induced contusion by electrically burning the cerebral cortex. After craniectomy in the manner described above, we inserted an iron wire-loop electrode with a diameter of 2 mm into the cerebral cortex through the exposed dura. We induced a 5-watt high-frequency current through the iron wire-loop electrode for 5 sec using an electric cautery (Hi-na dial termoMB122 bipolar scissors set, Kirikanyoukou Co., Ltd., Tokyo, Japan). This electrical burn did not invade the CA region. After the induction of brain concussion and contusion, we sutured the line of the scalp incision with silk braid (sterilized braid silk 4/0, Akiyama factory, Tokyo, Japan) without replacing the skull. We then allowed the rats to recover with a rectal thermometer and were maintained at 37°C using a heating pad. The rats were neither intubated nor ventilated during the operation.

Confirmation of the SBD following brain concussion and contusion by MRI

We performed magnetic resonance imaging (MRI) for all rats in this experiment to confirm the occurrence of SBD at 1, 48, and 72 hrs after the initial concussion or contusion using a superconducting 1.5 T MRI system (EXCELART Vantage™, Toshiba Medical systems, Tokyo, Japan). Before the MRI measurements were obtained, the rats were anesthetized via an intraperitoneal injection of sodium pentobarbital (40 mg/kg). We then placed the rats in a prone position with their heads fixed in a round surface coil (4 cm in diameter), and T2-weighted images were taken using a fast spin-echo (FSE) sequence (TR/TE = 4000/102) in the coronal plane with a section thickness of 3 mm at 1, 48, and 72 hrs after the initial insult (Kuwabara et al. 2002). We identified the lesion area as a T2 high-intensity area on MRI. We used rats that showed no or only extradural hemorrhage on the MRI at 1 hr after the initial concussion and a T2 high-intensity area invading the CA region at 48 and 72 hrs as the concussion model for the subsequent experiments (Kuwabara et al. 2002) (Fig. 1A and B). On the other hand, for the contusion model, we used rats that showed a T2 high-intensity area in the cerebral cortex alone corresponding to the initial 2-mm diameter electrical burn at 1 hr after the initial contusion and also showed expansion of the T2 high-intensity area into the CA region at 48 and 72 hrs (Sergienko et al. 1990) (Fig. 1C and D). The rats that did not show the above findings on MRI were excluded from the present study.

Histopathological analyses of the CA region by HE and TUNEL staining

Following MRI, the rats were killed via an intraperitoneal injection of sodium pentobarbital (200-400 mg/kg). We then perfused them through the heart with 0.9% physiological saline (200 ml) to remove blood cells and then 10% paraformaldehyde in 0.1 M PBS (pH 7.4, 200 ml) to fix their brains. Then, we removed the whole brains and preserved them in 500 ml of PFA-PB for 48 hrs at 4°C. The fixed brain tissues were embedded in paraffin and cut into coronal sections (5 μm thickness) at the level of the CA region (4 mm caudal to Bregma), and we stained the sections with HE (hematoxylin and eosin) to observe morphological changes and TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling) to detect DNA fragmentation in the CA region (Hong et al. 2002; Nag et al. 2005). We analyzed the sections using a light microscope (×400) (BH-2, Olympus, Tokyo, Japan) (Luo et al. 2002). To evaluate the extent of damage quantitatively, we counted the number of abnormal neuronal cells and TUNEL positive cells per 1 mm² of the CA region at the trauma site and calculated the percentage of abnormal neuronal cells and TUNEL positive cells in each group (Nakajima et al. 2004).

Quantification of caspase-3 activity by chemiluminescence

We analyzed the time course of caspase-3 activity after the initial brain insult. We removed the brain tissue at the site of injury at 1, 48, and 72 hrs after the initial insult and dissected the tissue into the cerebral cortex and the CA region under surgical loupe visualization. These tissues were immediately frozen in liquid nitrogen (−196°C) and thawed in a solution of 10% sodium dodecyl sulfate (SDS) and 20% polyoxyethylene (20) sorbitan monolaurate (Tween20). Then, we homogenized the tissues by ultrasound (20 Hz, 30 sec) (UH-50, SMT Co., LTD., Tokyo, Japan) and cooled them with crushed ice. The homogenates were centrifuged at 100,000 × g for 1 hr at 4°C, and the supernatant was analyzed with a protein quantification kit (Protein Quantification Kit, Dojindo Molecular Technologies, Tokyo, Japan). The homogenates were centrifuged at 100,000 × g for 1 hr at 4°C, and the supernatant was analyzed with a protein quantification kit (Protein Quantification Kit, Dojindo Molecular Technologies, Tokyo, Japan) and adjusted to 1,000 #/ml of protein with distilled water. The caspase-3 activity per 250 μl of this solution was detected using an assay kit (Caspase-Glo™/T Assay, Promega Co., LTD., Madison, U.S.A.) and a chemiluminescence-measuring instrument (BIOLUMAT LB 9505, BELTHOLD Co. LTD., Bad Wildbad, Germany) (Liu et al. 2004).
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Statistical analysis
All data were coded, recorded, and analyzed using commercially available statistical software packages. All of the data are represented as the mean ± S.D. or S.E. Differences between groups were compared by the Kruskal Wallis H-test, and the Mann-Whitney U-test and Bonferroni’s correction were used for post-hoc analyses. Differences were considered significant at \( p < 0.05 \).

Results
Histopathological changes in the CA region
We analyzed the histopathological changes in the CA region by HE staining (Fig. 2) and Tunnel staining (Fig. 3). The time-dependent changes in the frequency of abnormal neuronal cells are summarized in Fig. 4. In the concussion group, the percentage of abnormal neuronal cells that demonstrated vacuolation or nuclear condensation was increased at 1 hr after the insult (33.0 ± 8.2%) compared to that in the pre-injury group (0%) and was increased further at 48 hrs (48.1 ± 7.2%) (Fig. 2A, B, and E; Fig. 4). At 72 hrs, the percentage of abnormal neuronal cells (49.2 ± 10.5%) was similar to that at 48 hrs (Fig. 4, \( p < 0.01 \)). Likewise, as shown in Figs. 3A and B, TUNEL-positive neurons that showed vacuolation and nuclear condensation on HE-staining were present in the CA region of the concussion group at 1 hr (35.0 ± 9.7%), 48 hrs (49.8 ± 8.7%), and 72 hrs (51.3 ± 12.2%) after the concussion (\( p < 0.01 \)) (pre-injury: 0%; Fig. 3E). On the other hand, in the contusion group, there was only a small population of abnormal neuronal cells (11.7 ± 7.6%) and TUNEL-positive cells (9.6 ± 4.9%) in the CA region at 1 hr after the insult (\( p < 0.01 \)) (Fig. 2C and Fig. 3C). Subsequently, the frequency of abnormal neuronal cells (42.6 ± 9.9%) and TUNEL-positive cells (47.1 ± 4.5%) was increased at 48 hrs (\( p < 0.01 \)) (Fig. 2D and Fig. 3D). At 72 hrs, the percentages of abnormal neuronal cells (43.2 ± 3.3%) and TUNEL-positive cells (47.0 ± 5.2%) were similar to those seen at 48 hrs (Fig. 4). The results of HE staining were well correlated to those of TUNEL staining in both the concussion and contusion groups (Fig. 4).

Time-dependent changes in caspase-3 activity
The caspase-3 activity (c.p.m: count per minute) in the CA region of the concussion group increased to 94,183 ± 66,281 c.p.m./mg protein at 1 hr (pre-injury: 13,261 ± 5,045 c.p.m./mg protein) and decreased to 20,800 ± 2,719 c.p.m./mg protein by 48 hrs after the concussion (\( p < 0.01 \)) (Fig. 5A, dotted line). In the contusion group, the caspase-3 activity in the CA region increased slightly at 1 hr (25,171 ± 5,340 c.p.m./mg protein), and further increased to 68,228 ± 29,601 c.p.m./mg protein at 48 hrs after the contusion (\( p < 0.01 \)) (Fig. 5A, solid line). At 72 hrs after the initial injury, the caspase-3 activity decreased in both the concussion (1,800 ± 550 c.p.m./mg protein) and contusion (1,537 ± 962 c.p.m./mg protein) groups (\( p < 0.01 \)). On the other hand, the caspase-3 activity in the cortex of the concussion group increased to 79,157 ± 66,378 c.p.m./mg protein at 1 hr and then decreased at 48 hrs (20,931 ± 10,768 c.p.m./mg protein) and at 72 hrs (1,233 ± 470 c.p.m./mg protein) after the concussion (pre-injury: 19,101 ± 7,765 c.p.m./mg protein) (\( p < 0.01 \)) (Fig. 5B, dotted line). This time-dependent change was similar to the time course seen in the CA region of the concussion group. In the injured cortices of the contusion group, the caspase-3 activity did not change signifi-
Fig. 2. Histopathological changes in the CA region by HE staining.
Histopathological changes were analyzed by HE staining in the CA region from concussion (A, B), contusion (C, D) and pre-injury (E) rats. (A) 1 hr after concussion. The number of abnormal neuronal cells was increased compared to pre-injury (E). (B) 48 hrs after concussion. The abnormal neuronal cells increased further. (C) 1 hr after contusion. There were only a few abnormal neuronal cells. (D) 48 hrs after contusion. The abnormal neuronal cells increased and the number of abnormal neuronal cells was similar to that observed in concussion group (arrows: abnormal neuronal cells, vacuolated neuronal cells, or neuronal cells with nuclear condensation). Note that the histopathological changes at 72 hrs (not shown) are similar to those at 48 hrs (see Fig. 4).

Fig. 3. Detection of DNA fragmentation in the CA region by TUNEL staining.
DNA fragmentation was analyzed in the CA region by TUNEL staining from concussion (A, B), contusion (C, D) and pre-injury (E) rats. (A) 1 hr after concussion. The number of TUNEL-positive cells was increased compared to pre-injury (E). (B) 48 hrs after concussion. The number of TUNEL-positive cells was further increased. (C) 1 hr after contusion. There were only a few TUNEL-positive cells. (D) 48 hrs after contusion. The TUNEL-positive cells increased and the number of abnormal neuronal cells was similar to that observed in concussion group (arrows: TUNEL-positive cells). Note that the number of the TUNEL-positive cells at 72 hrs (not shown) is similar to that at 48 hrs (see Fig. 4).
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The number of abnormal neuronal cells and TUNEL-positive cells was counted per 1 mm² of the CA region. The HE staining results are well correlated to those of TUNEL staining. The difference between concussion and contusion was particularly obvious at 1 hr after the initial injury (Mann-Whitney U-test with Bonferroni’s correction after Kruskal Wallis H-test; vs. pre-injury, * p < 0.05; ** p < 0.01). Five animals were analyzed for each time point.

Fig. 5. The time-dependent changes in caspase-3 activity after traumatic brain injury.

Caspase-3 activity was quantified by chemiluminescence after concussion or contusion in the hippocampus (A) and cerebral cortex (B). In the CA region (A), the caspase-3 activity was increased at 1 hr and then decreased by 48 hrs after concussion (dotted line), while it was increased until 48 hrs after contusion (solid line). At 72 hrs after each brain injury, caspase-3 activity was decreased to the level that was lower than the pre-injury level. In the cerebral cortex (B), the caspase-3 activity was increased at 1 hr and decreased by 48 hrs after concussion (dotted line), while it remained unchanged at 1 hr and 48 hrs after contusion (solid line). The data are presented as the mean ± s.e.; six animals were analyzed for each time point. (Mann-Whitney U-test with Bonferroni’s correction after Kruskal Wallis H-test; vs. pre-injury; **p < 0.01).

Discussion

This study has revealed the difference in the time courses of apoptotic delayed cell death in the CA region between concussion and contusion. The apoptotic cell death
in the CA region is mostly induced at 1 hr after concussion and the process of apoptotic delayed cell death was completed by 48 hrs. On the other hand, the apoptotic cell death in the CA region is induced only slightly at 1 hr after contusion and progressed by 48 hrs. Moreover, in the CA region, the caspase-3 activity was increased at 1 hr after the concussion and decreased by 48 hrs, while the caspase-3 activity was increased only marginally at 1 hr after the contusion and significantly increased at 48 hrs. In both types of the brain injury, the caspase-3 activity in the CA region was decreased at 72 hrs after the insult. At 72 hrs after concussion or contusion, there may be only a small number of neuronal cells that express caspase-3. The changes in the caspase-3 activity were well correlated to the histopathological changes.

The differences between concussion and contusion may be linked to differences in the cause of the SBD. As it was reported that excessive extracellular release of glutamic acid is induced within 30 min after the initial injury and returns to its normal concentration by 45 min (Katoh et al. 1997), the main cause of SBD following concussion is a temporary increase in the glutamic acid concentration, which induces neuronal cell death (Tavazzi et al. 2005). On the other hand, downregulation of the glutamic acid receptor has been demonstrated after contusion (Miller et al. 1990). So a temporary increase in glutamic acid is not considered to be the main cause of SBD following contusion. After contusion, mechanical injuries, such as a disturbance of microcirculation, hemorrhage, or local brain edema, which induce secondary mechanical effects on capillary vessels and continue for a long time, are the main causes of SBD (Kuwabara et al. 2002; Nakajima et al. 2004). Taking these facts together, SBD caused by apoptotic cell death in the CA region is induced in the acute stage by concussion and in the transition period to the chronic stage by cortical contusion.

The present study indicates that any anti-apoptotic-cell-death treatment used to prevent SBD must be performed by 1 hr or at latest 48 hrs after concussion and by 48 hrs after contusion, and these treatments might be not effective at 72 hrs after the initial insult. On the other hand, the time course of caspase-3 activity in the cortex of the concussion group showed the same transitional pattern as in the hippocampus, but the caspase-3 activity in the cortex of the contusion group did not increase for 72 hrs. This suggests that brain contusion dose not induce apoptotic cell death in the area that was injured directly.

The pathological differences between brain concussion and contusion influenced the MRI findings: at 1 hr after concussion, no abnormal findings were found in the brain parenchyma while the only T2 intensity area was observed in the area of the cerebral cortex corresponding to the initial 2-mm diameter mechanical injury after contusion. It was reported that the mechanical injury was detected on MRI (Wilberger et al. 1987; Gentry et al. 1988), whereas biochemical abnormalities such as the excessive release of glutamic acid and intracellular enzymatic abnormalities are not detected on MRI (Schuhmann et al. 2003).

The present study indicates that the processes of SBD, induced by apoptosis, in the CA region differ between concussion and contusion; namely, the appropriate therapeutic period for SBD to suppress apoptosis differs between the two types of the primary brain injury. The best method of treatment depends on the type and severity of the brain injury, which can be determined by follow-up MRI, because there are several differences in the causative pathological mechanisms behind the apoptosis that causes SBD.

References


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