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Caspase-3 activation and lipid peroxidation are important for apoptotic cell death in cerebral infarction

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Abstract

To analyze the mechanism of apoptotic cell death in cerebral infarcts, we immunohistochemically and biochemically examined the lesions of thromboembolic cerebral infarction in a rat model, ranging from day 1 to 5 after induction. The number of TUNEL (terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick end-labeling)-positive cells was the highest in 2-day-old infarcts, when both activation of caspase-3 and up-regulation of HO-1 (inducible hemeoxygenese-1), an indicator of oxidative stress, also peaked. Moreover, the amount of lipid peroxides progressively increased until day 5. However, no activation of JNK (c-Jun N-terminal kinase) was found. Thus, apoptotic cell death can be caused by progressive increase of both lipid peroxidation and caspase-3 activity in cerebral infarction.

Introduction

Apoptotic cell death has been observed in various cerebral injuries. Injured neuronal and glial cells following chemical or physical stress signals release a wide variety of toxic molecules to their death in the lesions of cerebral infarction. Certain excitable amino acids released from injured cells, such as glutamate, can cause apoptotic cell death through an increased intracellular calcium concentration[1)]. Moreover, toxic cytokines such as tumor necrosis factor (TNF)-α, interleukin 1β (IL-1β) and IL-6 activate specific receptor-mediated signals, causing apoptotic cell death[2)]. In addition, Bcl-2 family proteins, inducible nitric oxide synthase (iNOS), cyclooxigenase-2 (COX-2) and superoxide dismutase (SOD) are well-known molecules, which have been expressed in the process of apoptotic cell death[3-5)]. Moreover, reactive oxygen species (ROS), caspases and c-Jun N-terminal kinase (JNK) have been noted in cerebral infarction although the detailed intracellular mechanisms to induce apoptotic cell death are not fully understood[6-8)].

An animal model that closely reflects cerebral infarction in humans is essential to define the molecular mechanism of the apoptotic cell death *in vivo*. A homologous thromoboembolic cerebral infarction rat model appears to be the most suitable, since this reliable and reproducible model is very similar in many aspects to those in humans. Moreover, it is easy to do correlative studies on morphological, biochemical, metabolic, and physiological parameters at selected time points to determine the development of cerebral infarction[9)10)].

We have reported previously that hydrogen peroxide or lipid peroxide is important for the activation of

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caspase-3 and apoptotic cell death[11)12)]. On the other hand, a free-radical scavenger, MCI-186, could apparently reduce the size and severity of edematous lesions of the same rat model[13)]. Moreover, administration of cyclophosphamide (CP), has induced an apparent delay of repair process in the same model[14)]. Thus, this study was focused on ROS and caspase-3 and designed to elucidate the mechanism of apoptotic cell death in the cerebral infarcts produced by the thromboembolic method. We show the molecular mechanisms to promote this cerebral infarction by using rat model.

Materials and Methods

1. Reagents

Antibodies raised against actin was purchased from SantaCruz Inc (California, USA). Antibodies (phospho-JNK and JNK) were obtained from Cell Signaling Inc (Danvers, MA, USA). Anti-HO-1 antibody was obtained from StressGen Biotechnologies Corporation (Hamburg, Germany). Acetyl-Asp-Glu-Val-Asp-(7-methoxycoumarin-4-yl) acetyl group (Ac-DEVD-MCA) peptide was purchased from the Peptide Institute (Osaka, Japan).

2. Preparation of animals

Cerebral infarction was made as described previously[9)]. Briefly, male Wistar strain rats were anesthetized with ether, and 0.1 ml of blood was obtained by cardiac puncture and stored at room temperature for 2 days. A 0.2 ml clot suspension with fragments of varying sizes was used for the embolization. Animals were then anesthetized again, and the left common carotid artery was surgically exposed. The emboli suspended in the saline were injected into the common carotid artery. For the control sham study, instead of blood clot emboli, 0.2 ml of saline was injected. The animals were killed under deep anesthesia by transcardiac perfusion fixation at 1, 2, 3, 5, and 8 days after embolization. In addition, the animals used for immunoblot analysis were killed without transcardiac perfusion fixation at the same time course under deep anesthesia. This project was approved and followed the guidelines determined by the Animal Care and Use Committee of Tokyo Medical University.

3. Tissue preparation

The brains were removed, cut coronally and embedded in paraffin, then, 3 μm-thick serial sections were made and each section was stained by hematoxylin-eosin (HE).

4. Quantification of lipid peroxidation

After preparation of the brains, the amount of lipid peroxide in the brain was quantified using a Lipid Hydroxide Assay kit (Cayman Chemical Co., USA). The data were obtained by following the manufacturer's instructions as described previously[11)]. Briefly, lysate of the brain was collected in lysis buffer containing 10 mM Tris-HCl (pH 7.6), 150 mM NaCl and 1 mM phenylmethylsulfonyl fluoride (PMSF) using a teflon homogenizer at 60 rotations per minute. Then, ExtractR in the assay kit solution saturated with methanol and chloroform was added to each sample, and lipid peroxides were extracted from the chloroform layer after centrifugation. Chromogen (2.25 mM ferrous sulfate and a 1.5% methanolic solution of ammonium thiocyanate) was mixed with each sample, and the hydrophobic chloroform solution was extracted after centrifugation. To measure the amount of lipid peroxide, tinctorial chloroform solutions were analyzed with a colorimeter (Viento, BT-MQX200DN, Dainippon-pharm, Osaka, Japan) at 500 nm. The obtained values of absorbance were calibrated to the amount of total protein of rat brain. The data were calculated as $\{$(sample value)$-$(blank value)/(untreated sample value)$-$(blank value)$\} \times 100$.

5. Measurement of caspase activity

The caspase-3 activity was measured as described previously with a brief modification[11)12)15)]. Samples from rat brain were lysed in a buffer containing 10 mM Hepes-KOH (pH 7.4), 2 mM ethylenediamine tetraacetic acid (EDTA) and 1 mM PMSF. Once centrifuged, a volume of supernatant was mixed with 2$\times$ICE buffer containing 20 mM Hepes-KOH (pH 7.4), 20% glycerol (v/v), 2 mM PMSF and 50 μM Ac-DEVD-MCA and incubated at 37°C for 1 hour. We had already confirmed that this reaction time for the measurement of caspase-3 activity was included in the linear range. After the addition of 200 μl of distilled water, the fluorescence was detected using a spectrofluorometer (Fluoroskan Ascent, L-5210420, Dainippon-pharm, Osaka, Japan) at 355 nm excitation and 460 nm emission. The data obtained were normalized with respect to the quantity of protein in the lysate.

6. TUNEL staining

For identification of apoptotic cells, we used a terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) method (DNA fragmentation detection kit, Promega, Madison, WI, USA) according to the manufacturer's instructions[15)]. Briefly, the deparaffinized and rehydrated sections were reacted with the terminal deoxynucleotidyl transferase using fluorescent nucleotides at 37°C for 1 h after treatment with proteinase K, and then the reaction was terminated by adding 2$\times$saline-sodium citrate (SSC). Apoptotic nuclei were observed and photographed with a fluorescence microscopy system (TE2000-U, Nikon, Tokyo, Japan).

7. Western blotting

The ipsilateral injured brains were homogenized using a polytron on ice in lysis buffer 1% (sodium dodecyl

sulfate (SDS), 50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.5 mM sodium orthovanadate, 1 mM PMSF, 1 mM EDTA and 25 μg/mL of leupeptin). The homogenates were centrifuged at 14,000×g, and then supernatants were transferred to a fresh tube for further centrifugation (14,000×g). The clear lysates obtained were dissolved in sample buffer (70 μg of protein/sample), loaded for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) after the protein assay, and blotted onto a polyvinylidene fluoride membrane (Millipore, Massachusetts, USA) using a semi-dry electrophoretic transfer system (AE-6677, Atto, Tokyo, Japan). Then, the first specific antibody (1 : 1000 dilution) was loaded onto the membrane after blocking with BlockAce™ (Dainipponpharm, Osaka, Japan) at 4°C overnight and horse radish peroxidase-conjugated signal was enhanced using an avidin-biotin peroxidase complex (ABC) kit (Vecta Stain kit, Vector Laboratories, Burlingame, CA, USA) after the second antibody (diluted at one drop to 10 ml of PBS following the manufacturer's instructions). The bands were detected by enhanced chemiluminescence (ImmunoStar™ Reagents, Wako, Osaka, Japan) and visualized with a light-capture system (AE6962N, Atto).

8. Statistical evaluation

All values in the figures were expressed as the mean± SEM. Student's t-test was used to compare means between groups. A p value of less than 0.05 was considered to indicate a statistically significant difference.

Results

1. Apoptotic cell death in cerebral infarction

In the present model, the central parts of lesions underwent complete tissue destruction at day 5 (Fig. 1). TUNEL-positive cells were clearly found in the peripheral regions on 2- and 5-day-old infarcts (Fig. 2). The

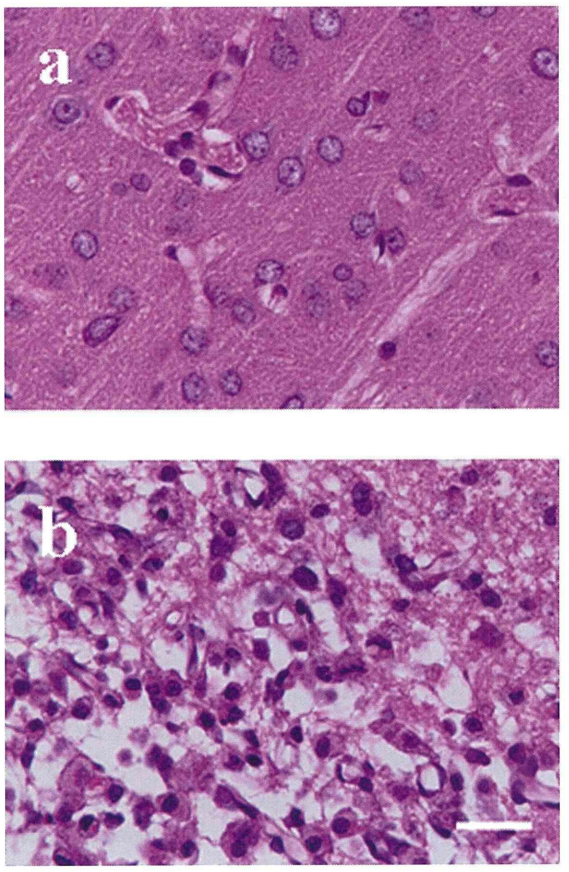

Fig. 1 The cell damage in the infarct model system. (a) sham brain at day 5, (b) infarcted brain at day 5. The operation was described in Materials and Methods. Bar represents 20 μm. The data represent three independent experiments.

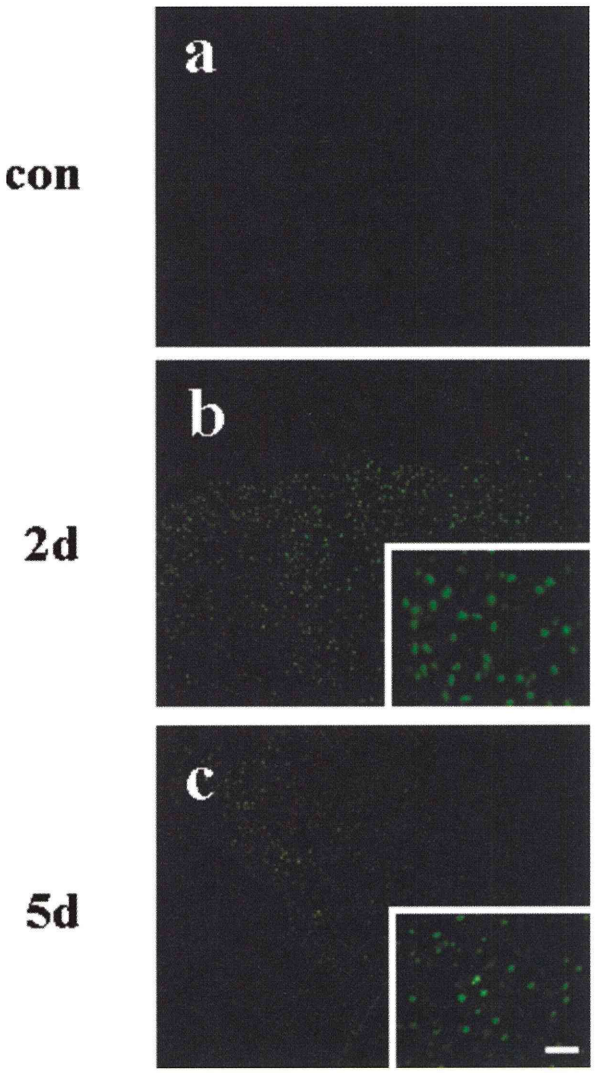

Fig. 2 Nuclear DNA fragmentation in thromboembolic infarction. Cells that died by apoptosis in the homologus thromboembolic infarction were identified using the TUNEL method as described in Materials and Methods. (a) sham at day 2, (b) infracted cortical lesion at day 2 or (c) 5 days of infarction. Bar represents 50 μm. Fig. 2a, 2b and 2c represent the same magnitude. The photographs of 5 times larger scale is interpolated in Fig. 2b and 2c.

number of TUNEL-positive cells was more prominent on 2-day-old lesion as compared with that on 5-day-old lesion (Figs. 2b and 2c).

2. Lipid peroxidation and activation of caspase-3

To clarify the possible stimuli leading to apoptosis-promoting factor(s), the ipsilateral brain tissue was subjected to measurement of the activation of caspase-3 and the amount of lipid peroxides. The activity of caspase-3, a cell death-promoting cysteine protease, was increased and maximal activation was observed at 2 days after occlusion (Fig. 3a). The lipid peroxides increased time-dependently up to the 5th day (Fig. 3b). It is known that HO-1 reflects the existence of oxidative stress accompanied with the production of reactive oxygen species (ROS). To investigate whether the oxidative stress was involved or not, we performed western blotting using anti-HO-1 antibody. As shown in Fig. 4, the expression of HO-1 remarkably increased from 1 day to 2 days after occlusion, however, c-Jun N-terminal kinase (JNK) which is also known as a cell death-promoting protein was not activated.

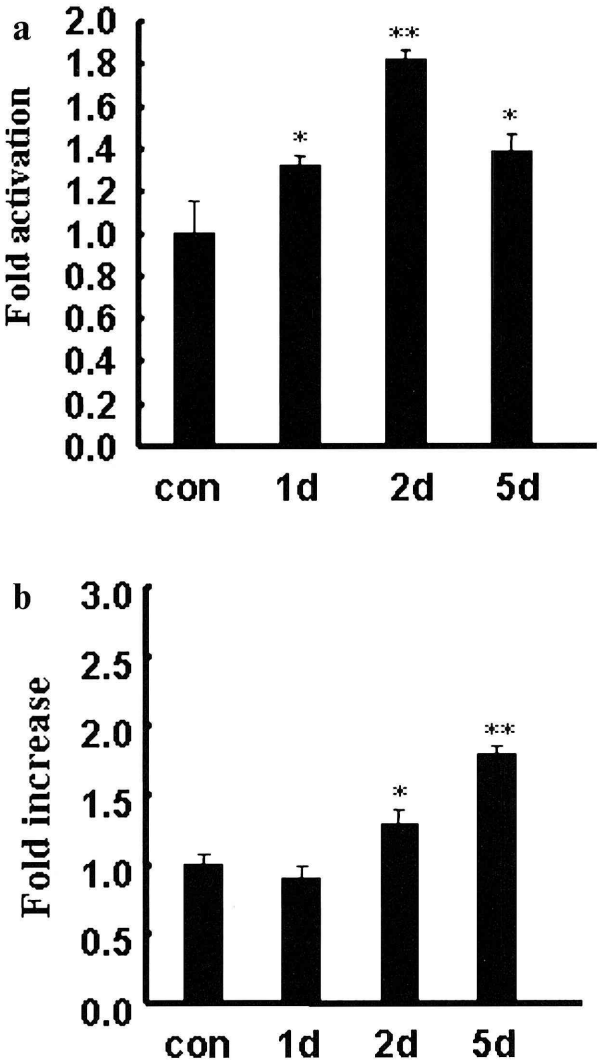

Fig. 3 The possible cell damage-promoting factors in infarction. (a) The activity of caspase-3 was measured as described in Materials and Methods. The data obtained show an increased ratio against the value from the sample of untreated rat (as a control). Values are the mean±SEM ($n=3$). $^{*}P<0.05$ or $^{**}P<0.01$. (b) The increase of lipid peroxides was measured. The data show an increased ratio against the value from the sample of untreated rat (control). Values are the mean±SEM ($n=3$). $^{*}P<0.05$, $^{**}P<0.01$

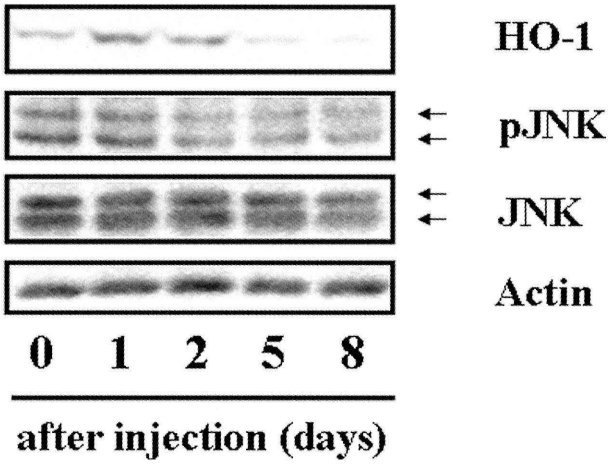

Fig. 4 Analysis of involvement of oxidative stress by detection of HO-1. The cerebral infarct sample was used for immunoblot analysis as described in Materials and Methods, and specific bands of HO-1, phospho-JNK (pJNK), JNK, and actin were detected. Actin was detected using the same sample for evaluation of uniformity per lane. Arrows indicate corresponding bands of isoforms of JNK. The data represent two independent experiments.

Discussion

We found in the present study that caspase-3 activity was strongly increased at day 2. Lipid peroxidation was also increased gradually and progressively. Moreover, JNK, a key molecule of apoptotic cell death, has not been activated throughout the experiment as same as other's report[8]. These data clearly indicate that both caspase-3 and lipid peroxidation play important roles in apoptotic cell death in cerebral infarcts without participation of the JNK activity. Thus, it is possible to consider that there are at least two independent pathways contributing to apoptotic cell death in cerebral infarction. A possible oxidative stress may be constituent for the pathways because the expression of HO-1 was observed from 1 and 2 day-old lesions. It has been reported that lipid peroxides are important to induce apoptotic cell death[16]. The other possible pathway may also induce apoptotic cell death because the peak caspase-3 activity was observed at day 2. Thus, there may be a role of peroxidation signaling and caspase signaling in the promotion of cell damage in cerebral

infarcts.

Although the exact mechanism of apoptotic cell death by lipid peroxidation is remains unclear in this study, a possible pathogenesis can be considered. As suggested using the same animal model[7], released lipids from cell debris in the central necrotic areas progressively accumulate centrifugally along the border of the infarcts to construct a distinct lipid layer at day 2. Then, macrophages clean up the debris and transform into foamy macrophages filled with lipids. Finally, dying foamy macrophages release the lipids and undigested debris. During these periods, lipid peroxides must be also produced continuously, resulting in the gradual increase of lipid peroxides in the lesions. Likewise, reactive microglia and astrocytes release a wide variety of chemokines, cytokines and trophic factors in and around the lesions. One of them is TNF-α, which has a potential to induce apoptotic cell death[2)17)]. Moreover, TNF-α can promote activation of caspase-3, which may be released from 2 days after episode[17)].

In the present study, we showed that the number of TUNEL-positive cells was abundant at day 2 and then decreased on day 5 in the peripheral regions. Thus, apoptotic cell death occurred quickly within a few days after embolization. In general, hippocampal neurons have higher vulnerability to ischemia, as compared with those in the cerebral cortex. In this model, we believe that many hippocampal neurons may be stained with TUNEL method. There may be a common phenomenon to induce apoptotic cell death in the infarction after embolization.

As described in Results, JNK was not activated in this study. Indeed, there is evidence that JNK is not involved in the pro-apoptotic cell death[8)18)]. If so, p38 mitogen-activated protein kinase (MAPK) may play an important role in infarction because either JNK or p38 MAPK belongs to the same functional pro-apoptotic kinase[6)19)]. Furthermore, we need to consider the involvement of a caspase cascade. Since caspase-3 is activated via caspase-9 or -12 without release of cytochrome c from mitochondria[20)], the caspases and/or cytochrome c could be involved in the development of cerebral infarction. Although the importance of the caspase-12 remains controversial in apoptotic cell death[21)22)], an activation of caspase-12 by induction of endoplasmic reticulum stress has been reported in cerebral infarcts[23)]. Interestingly, the active fragment of caspase-12 sorts into nucleus, suggesting that the expression of unidentified important genes occurs by the interaction between the promoter region of the genes and the active fragment of caspase-12[24)]. Thus, we need to analyze further the mechanism of the possible involvement of caspase-12 in apoptotic cell death in cerebral infarction.

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References

1) Tymianski M, Wallace MC, Spigelman I, Uno M, Carlen PL, Tator CH, Charlton MP : Cell-permeant Ca^{2+} chelators reduce early excitotoxic and ischemic neuronal injury in vitro and in vivo. Neuron **11** : 221-235, 1993.
2) Iadecola C : Mechanisms of cerebral ischemic damage in cerebral ischemia. Molecular and Cellular Pathophysiology, Humana Press, NJ, pp. 3-32, 1999.
3) White BC, Sullivan JM, DeGracia DJ, O'Neil BJ, Neumar RW, Grossman LI, Rafols JA, Krause GS : Brain ischemia and reperfusion : molecular mechanisms of neuronal injury. J Neurol Sci **179** : 1-33, 2000.
4) Nogawa S, Zhang F, Ross E, Iadecola C : Cyclooxigenase-2 gene expression in neurons contributes to ischemic brain damage. J Neurosci **17** : 2746-2755, 1997.
5) Kinouchi H, Kamii H, Mikawa S, Epstein CJ, Yoshimoto T, Chan PH : Role of superoxide dismutase in ischemic brain injury : a study using SOD-1 transgenic mice. Cell Mol Neurobiol **18** : 609-620, 1998.
6) Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME : Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science **270** : 1326-1331, 1995.
7) Jenner P : Oxidative damage in neurodegenerative diseases. Lanset **344** : 796-798, 1994.
8) Gunn-Moore FJ, Tavaré JM : Apoptosis of cerebellar granule cells induced by serum withdrawal, glutamate or beta-amyloid, is independent of Jun kinase or p38 mitogen activated protein kinase activation. Neurosci Lett **250** : 53-56, 1998.
9) Kudo M, Aoyama A, Ichimori S, Fukunaga N : An animal model of cerebral infarction : Homologous blood clot emboli in rats. Stroke **13** : 505-508, 1982.
10) Kudo M, Nagayama T : Light and electron microscopic study of lipid accumulation along margins of experimental cerebral infarcts in rats. Stroke **19** : 1544-1549, 1988.
11) Shimoke K, Kudo M, Ikeuchi T : MPTP-induced reactive oxygen species promote cell death through a gradual activation of caspase-3 without expression of GRP78/Bip as a preventive measure against ER stress in PC12 cells. Life Sci **73** : 581-593, 2003.
12) Shimoke K, Kudo M, Ikeuchi T : Opposing mecha-

nism of extracellular signal-regulated kinases on hydrogen peroxide-induced apoptosis in PC12 cells. J Tokyo Med Univ **65**: 283–289, 2007.

13) Niyaz M, Numakawa T, Matsuki Y, Kumamaru E, Adachi N, Kitazawa H, Kunugi H, Kudo M: MCI-186 prevents brain tissue from neuronal damage in cerebral infarction through the activation of intracellular signaling. J Neurosci Res **85**: 2933–2942, 2007.

14) Feng Y, Kudo M, Shimoke K, Ikeuchi T, Ebihara Y, Takasaki M: Suppression of angiogenesis causes a significant delay of repair process in rat thromboembolic cerebral infarction. J Tokyo Med Univ **60**: 489-500, 2002.

15) Shimoke K, Chiba H: Nerve growth factor prevents 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced cell death via the Akt pathway by suppressing caspase-3-like activity using PC12 cells: Relevance to therapeutical application for Parkinson's disease. J Neurosci Res **63**: 402–409, 2001.

16) El Kossi MM, Zakhary MM: Oxidative stress in the context of acute cerebrovascular stroke. Stroke **31**: 1889–1892, 2000

17) Zaremba J, Skrobanski P, Losy J: Tumour necrosis factor-alpha is increased in the cerebrospinal fluid and serum of ischaemic stroke patients and correlates with the volume of evolving brain infarct. Biomed Pharmacother **55**: 258–263, 2001.

18) Shimoke K, Amano H, Kishi S, Uchida H, Kudo M, Ikeuchi T: Nerve growth factor attenuates endoplasmic reticulum stress-mediated apoptosis via suppression of caspase-12 activity. J Biochem **135**: 439–446, 2004.

19) Hattori H, Shibata M, Sugaya T, Hamada J, Fukuuchi Y: Delayed phosphorylation of p38 mitogen-activated protein kinase in the AT1a knock-out mouse striatal neurons during middle cerebral artery occlusion and reperfusion. Neurosci Lett **341**: 9–12, 2003.

20) Rao RV, Castro-Obregon S, Frankowski H, Schuler M, Stoka V, del Rio G, Bredesen DE, Ellerby HM: Coupling endoplasmic reticulum stress to the cell death program. An Apaf-1-independent intrinsic pathway. J Biol Chem **277**: 21836–21842, 2002.

21) Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J: Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature **403**: 98–103, 2000.

22) Di Sano F, Ferraro E, Tufi R, Achsel T, Piacentini M, Cecconi F: Endoplasmic reticulum stress induces apoptosis by an apoptosome-dependent but caspase 12-independent mechanism. J Biol Chem **281**: 2693–2700, 2006.

23) Shibata M, Hattori H, Sasaki T, Gotoh J, Hamada J, Fukuuchi Y: Activation of caspase-12 by endoplasmic reticulum stress induced by transient middle cerebral artery occlusion in mice. Neuroscience **118**: 491–499, 2003.

24) Fujita E, Kouroku Y, Jimbo A, Isoai A, Maruyama K, Momoi T: Caspase-12 processing and fragment translocation into nuclei of tunicamycin-treated cells. Cell Death Differ **9**: 1108–1114, 2002.

ラット自家血塞栓性脳梗塞巣におけるアポトーシス機構ではカスパーゼ-3の活性化と脂質過酸化の役割が重要である

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［目的］脳梗塞の病巣形成におけるアポトーシス機構を把握する。［方法］ラット自家血塞栓性脳梗塞モデルを使い，脳梗塞を作製した。カスパーゼ-3，過酸化脂質，あるいは酸化ストレスなどの分子生物学的パラメータを用い，さらに，免疫組織化学染色法，western blotting 法にて 5 日目までの病巣を経時的に解析した。［結果］2 日目，一過性のカスパーゼ-3 の活性化と病巣細胞中の核内 DNA の断片化が見られた。過酸化脂質量は 5 日目まで上昇し続けた。この期間，酸化ストレスによる過酸化物，ヘムオキシゲナーゼ-1(HO-1) の出現も確認したが，或る特定のアポトーシス進行に重要な因子とされる c-Jun N-terminal kinase (JNK) の活性化には変化がなかった。［結論］本動物モデルによる脳梗塞では，カスパーゼ-3 の活性化を伴う，2 日目をピークとしたアポトーシス機構と過酸化脂質の両者の関与が病巣形成に重要な役割を果たしていることが示唆された。

〈キーワード〉 ラット自家血塞栓性脳梗塞，カスパーゼ，過酸化脂質，ヘムオキシゲナーゼ，アポトーシス