hydroxyacyl-CoA dehydrogenase (SCHAD): additionally, ABAD catalyzed a wide spectrum of substrates, including steroids, cholic acids and fatty acid (Yang et al., 2005). Thus, if this multifunctional enzyme would have an additional function, it might be reasonable. When we pay attention on fatty acid β-oxidation (Schulz, 1991), the β-oxidation is not available in energy metabolism in the brain (Penicaud et al., 2006); however; ABAD, an enzyme involved in β-oxidation, expresses in the brain (Yang et al., 2005). Thus, it suggests that ABAD plays an alternative role in the brain instead of energy metabolism.

ABAD can detoxify 4-HNE only in the presence of NADH as a cofactor in the healthy brain according to our model. Thus, when energy metabolism to generate NADH is declined, the detoxification system by ABAD would not be functional, leading to amplifying toxic aldehydes. Since it is known that energy metabolism is poor in the brain of AD, NADH must be not abundant in AD brains. Moreover, in our model, Aβ plays a role toward the accumulation of 4-HNE by inhibiting the ABAD activity in the development of AD. Since 4-HNE stimulates the Aβ production (Tamagno et al., 2005), Aβ would in turn enhance to increase by 4-HNE. This vicious cycle could increase Aβ as well as 4-HNE, both of which should contribute to the pathogenesis of AD. Further study will be required to reveal the relationship between AD and ABAD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2007.07.002.

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BRAIN RESEARCH

Research Report

Involvement of mitoK$_{ATP}$ channel in protective mechanisms of cerebral ischemic tolerance

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ABSTRACT

Little work has been performed to determine roles of mitochondrial ATP-sensitive potassium channels (mitoK$_{ATP}$) in ischemic preconditioning (IPC) in brain. To investigate the role on cerebral IPC, we examined effect of 5-hydroxydecanoate (5-HD), a selective mitoK$_{ATP}$ blocker, and diazoxide (DZX), a selective mitoK$_{ATP}$ opener on various IPC models. An IPC model with gerbil: 2 min bilateral common carotid arteries occlusion (BLCO) + 24 h recovery + 5 min BLCO. 5-HD, DZX, vehicle was administered 30 min before 5 min BLCO. Seven days later, surviving CA1 neurons were counted. A focal IPC model with rat: 15 min middle cerebral artery occlusion (MCAO) + 48 h recovery + 90 min MCAO. Twenty-four hours before 90 min MCAO, 5-HD, DZX, or vehicle was administered. One day after 90 min MCAO, neurological symptoms and infarct volumes were evaluated. An in vitro IPC model with primary neuronal cultures: 8 min oxygen–glucose deprivation (OGD) + 24 h recovery + 70 min OGD. Thirty minutes before 70 min OGD, 5-HD or DZX were added. One day later, surviving neurons were counted. Mitochondrial membrane potential was also monitored. 5-HD significantly attenuated the protective effect of IPC in gerbil model, rat model, and in vitro OGD model. DZX significantly facilitated the protective effect of IPC in gerbil and rat model. The mitochondrial membranes were depolarized with IPC, and 5-HD treatment significantly reduced this effect. These results strongly suggest that mitoK$_{ATP}$ channel activation plays a key role in development of a protective mechanism of cerebral IPC.

1. Introduction

There is a continuous interest in understanding mechanisms of ischemic cell damage and in developing adequate treatments for stroke patients. It has been reported that ischemic tolerance (preconditioning) phenomenon, in which brief episodes of ischemia protect against subsequent lethal ischemia, involves endogenous cellular protective mechanisms. This phenomenon has been observed in various animal models of forebrain ischemia (Kitagawa et al., 1990, Kirino, 2002, Heurteaux et al., 1995) and focal cerebral ischemia (Chen et al., 1996, Barone et al., 1998, Puisieux et al., 2000, Shimizu et al., 2001, Nakamura et al., 2002). It was also observed in human stroke patients that transient ischemic attack (TIA) could

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protect against subsequent final stroke damage (Wegener et al., 2004). Among the various mechanisms considered as mediators of preconditioning in brain, considerable attention has been drawn to the activation of adenosine A1 receptors and ATP-dependent potassium (K_{ATP}) channels. This study line was influenced by the report that both the K_{ATP} channel blocker glibenclamide and the A1 receptor antagonist DPCPX (1,3-dipropyl-8-cyclopentylxanthine) could abolish the protective effect of preconditioning in a rat global ischemia model when administered during the preconditioning insult (Heurteaux et al., 1995). However, recent study failed to support a direct involvement of A1 receptors or plasma membrane K_{ATP} channels during early stages in the development of ischemic tolerance in vivo (Sorimachi and Nowak, 2004), stating that the role of mitoK$_{ATP}$ channels remained to be elucidated. Activation of mitochondrial ATP-sensitive potassium (mitoK$_{ATP}$) channels has been proposed to play a pivotal role in preconditioning (O'Rourke, 2000, Oldenburg et al., 2002) in heart. Pharmacological agents that open mitoK$_{ATP}$ channels showed preconditioning in heart (Garlid et al., 1997, Szewczyk and Wojtczak, 2002). Moreover, the physiological or chemical preconditioning phenomenon is prevented by selective mitoK$_{ATP}$ channel blockers (Horiguchi et al., 2003, Yoshida et al., 2004). The beneficial effects of mitoK$_{ATP}$ channel opener diazoxide have been also demonstrated in the heart (Garlid et al., 1997, O'Rourke, 2000, Oldenburg et al., 2002) and other organs (Kullin et al., 2003, Roth et al., 2006). However, in vivo experiments showing involvement of mitoK$_{ATP}$ channels in brain ischemic preconditioning were quite limited (Yoshida et al., 2004). Although they showed the cancellation of ischemic tolerance phenomenon by administration of mitoK$_{ATP}$ channel blocker 5-hydroxydecanoate (5-HD) in a rat MCAO model, because they administered 5-HD at 30 min before lethal ischemia, possibilities of direct effect of 5-HD itself still remain to be solved. In addition, we have published a report on the roles of adenosine receptors in ischemic tolerance phenomenon (Hiraide et al., 2001) and we were interested in clarifying the downstream signal transduction after adenosine receptor activation. To that end, we studied the roles of mitoK$_{ATP}$ channels in ischemic preconditioning by using mitoK$_{ATP}$ channel inhibitor 5-HD and mitoK$_{ATP}$ opener diazoxide (DZX) in a gerbil global ischemia model, rat transient middle cerebral artery occlusion (MCAO) model, and in vitro primary neuronal cell culture. Especially, in rat MCAO model, we administered 5-HD, or DZX at 24 h before the lethal ischemia (and 24 h after the preconditioning ischemia) to be free from the drug's direct effects on the lethal ischemia.

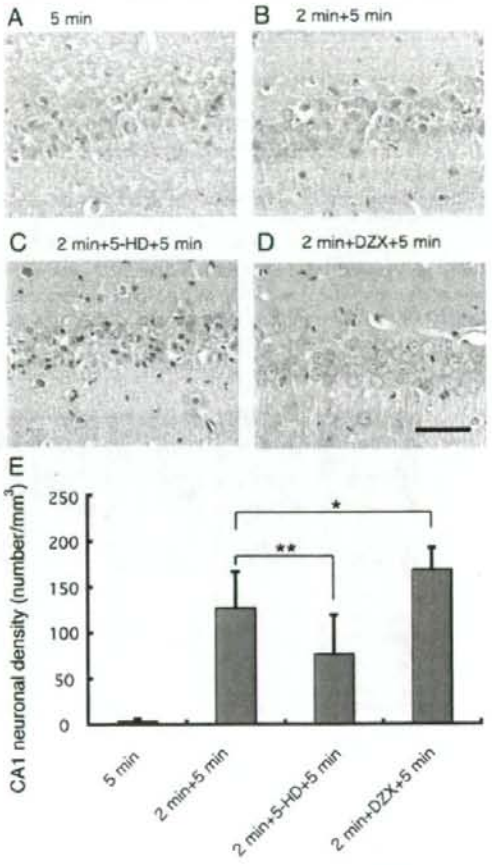

Fig. 1 – Five minutes of carotid artery occlusion gave rise to almost complete cell death (5 min; A). When 2 min ischemia was given 24 h before the 5 min ischemia (2 min+5 min; B), the surviving neurons were significantly enhanced. The increase was significantly reduced when 5-HD was administered before the 5 min ischemia (2 min+5HD+5 min; C). The increase was significantly enhanced when DZX was administered before the 5 min ischemia (2 min+DZX+5 min; D). Upper panels (A–D) showed typical histological appearances in gerbil hippocampus. Lower panel (E) showed CA1 neuronal densities. **: $p<0.01$, *: $p<0.05$. Scale bar in panel D shows 200 μm.

2. Results

2.1. *Transient global ischemia with gerbil*

Gerbil hippocampal CA1 neuronal densities after 5 min ischemia are shown in Fig. 1. Sham operation only showed neuronal densities of 187.3±3.5 (n=6). Without preconditioning ischemia, 5 min of carotid occlusion decreased survival cells down to close to zero (5 min group). In the groups with 2 min preconditioning ischemia at 24 h before the 5 min ischemia, the decrease of the number of surviving cells was significantly attenuated after 5 min of carotid occlusion (2 min+5 min group). When 5-HD was administered intraperitoneally 30 min before 5 min occlusion (2 min+5HD+5 min group), the number of surviving neurons was significantly decreased compared to the number in the 2 min+5 min group. On the other hand, when DZX was administered 30 min before 5 min ischemia (2 min+DZX+5 min group), the number of surviving neurons was significantly enhanced against the number of 2 min+5 min group.

2.2. *Focal cerebral ischemia with rat*

Physiological parameters (Blood pressure, pH, PO_2, PCO_2, blood sugar, body temperature, skull temperature) before, during and after ischemia were monitored. These data were within the normal range and no significant difference between groups was observed.

The volumes of rat cerebral infarction are shown in Fig. 2B. Fifteen minutes of MCA occlusion given 48 h before 90 min MCA occlusion gave rise to a significant reduction of total, cortical and striatal infarct volumes. When 5-HD was administered 24 h after the 15 min of MCA occlusion, the reduction of infarct volume was significantly attenuated. When DZX was administered 24 h after the 15 min of MCA occlusion, the reduction of infarct volume was significantly accelerated, except in the striatum. Similar tendencies were observed in rotametric test, grip strength (Fig. 2C), and neurological deficit score (Fig. 2D).

2.3. *In vitro cell analysis*

To mimic ischemia in vitro, we subjected cultured cortical neurons to OGD and incubated them under nitrogen gas, followed by reperfusion with medium containing O_2 and glucose. After treatment of neurons with OGD, almost 80% of the cells died (Fig. 3). When neurons were exposed to short-

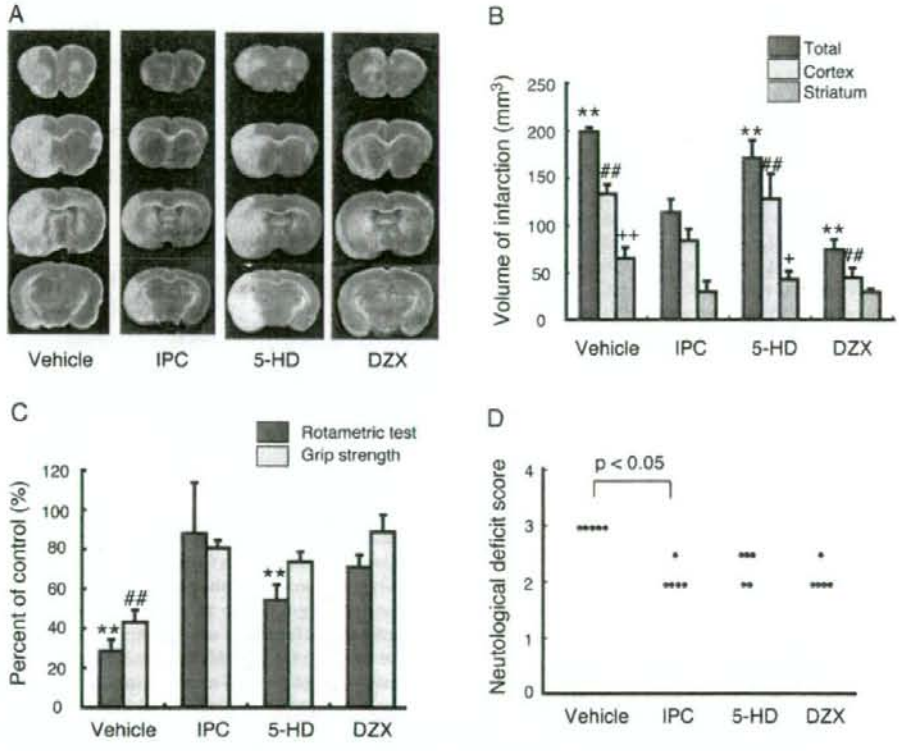

Fig. 2 – Panel A shows typical TTC staining at 24 h after 90 min of middle cerebral artery (MCA) occlusion. Rat cerebral infarct volume after 90 min of MCA occlusion is shown in panel B. Cortex, striatum, and total infarct volumes were evaluated. Fifteen minutes of MCA occlusion given 48 h before 90 min of MCA occlusion gave rise to a significant reduction of infarct volumes (IPC). When 5-HD was administered 24 h after the 15 min of MCA occlusion, the reduction of infarct volume was significantly attenuated (5HD). When DZX was administered 24 h after 15 min of MCA occlusion, the reduction of infarct volume was significantly accelerated, except in striatum (DZX). **: $p<0.01$ against total infarct volume of IPC, ##: $p<0.01$ against cortical infarct volume of IPC, +: $p<0.05$, ++: $p<0.01$ against striatal infarct volume of IPC. Panel C shows percent control values of rotametric test and grip strength. IPC groups showed significantly improved rotametric value and grip strength (IPC). When 5-HD was administered, the improvement in rotametric value was cancelled, however grip strength was not affected (5HD). DZX administration did not affect rotametric test and grip strength (DZX). **: $p<0.01$ against IPC value of rotametric test. ##: $p<0.01$ against IPC value of grip strength. Panel D shows neurological deficit scores. IPC groups showed significantly improved scores against vehicle (no-preconditioning) treated rats. 5-HD pre-treatment showed slight tendency of aggravation of the score (5HD). DZX pre-treatment did not affect the neurological score (DZX).

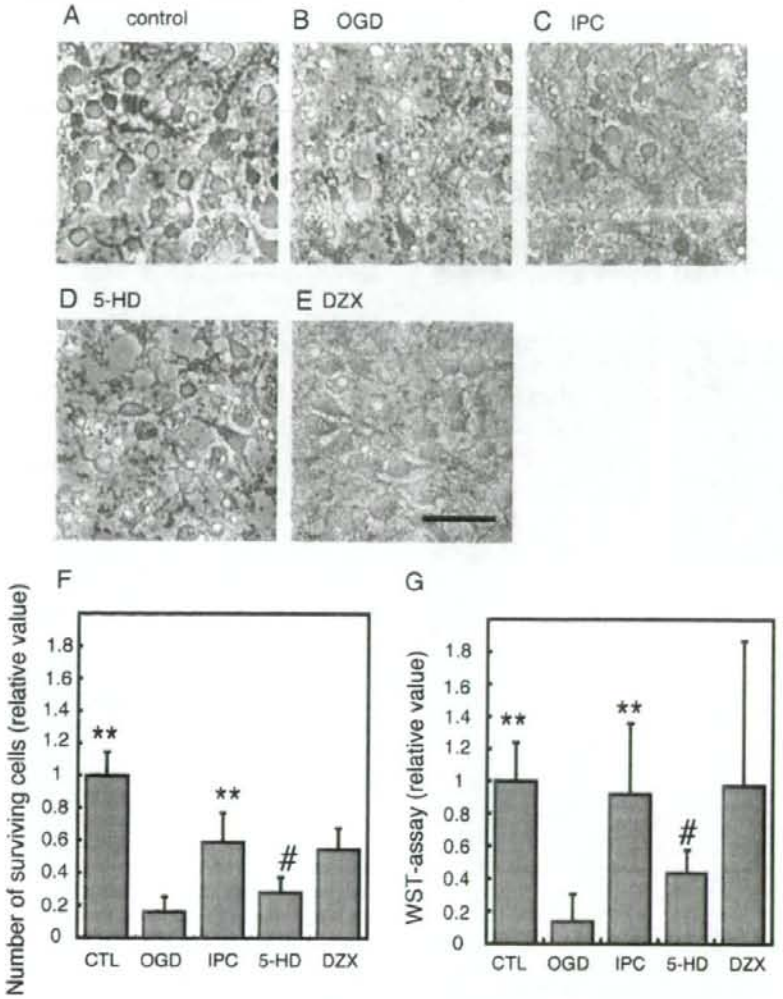

Fig. 3 – The numbers of surviving cells after OGD treatment and results of WST-1 assay were shown in panels F and G, respectively. Surviving neuronal counts were calculated as % of average of control value. The results of WST-1 assay were calculated as % of average of IPC value. OGD gave rise to more than 80% of cell death (OGD). When cells were pre-exposed to short-term OGD (preconditioning) before the lethal OGD, the numbers of surviving neurons were significantly increased ($p<0.01$, IPC). When cells were pre-treated with 5-HD, the increase was significantly attenuated ($p<0.05$, 5-HD); however, pre-treatment with DZX did not affect the surviving neurons (DZX). Upper panels (A–E) showed typical cell appearances in each groups. **: $p<0.01$ against values of OGD group. #: $p<0.05$ against values of IPC group. Scale bar in panel E shows 50 μm.

term OGD, the numbers of surviving cells significantly increased. However, when 5-HD was added to the medium 30 min before treatment with OGD, the increase was significantly cancelled. On the other hand, the addition of mitoK_{ATP} opener DZX did not affect the increased neuronal survival rate induced by IPC.

To assess the direct effect of IPC on mitochondrial function, we investigated mitochondrial membrane potential with TMRM (Fig. 4). When cells were exposed to IPC before lethal OGD, the TMRM intensities after OGD were significantly reduced, showing depolarization of mitochondrial membrane. When cells were pre-exposed to 5-HD, the reduction of TMRM intensities was significantly attenuated, indicating that the depolarization of mitochondrial membrane with IPC is partially due to the activation of mitoK_{ATP}. On the other hand, when DZX was pre-loaded to the

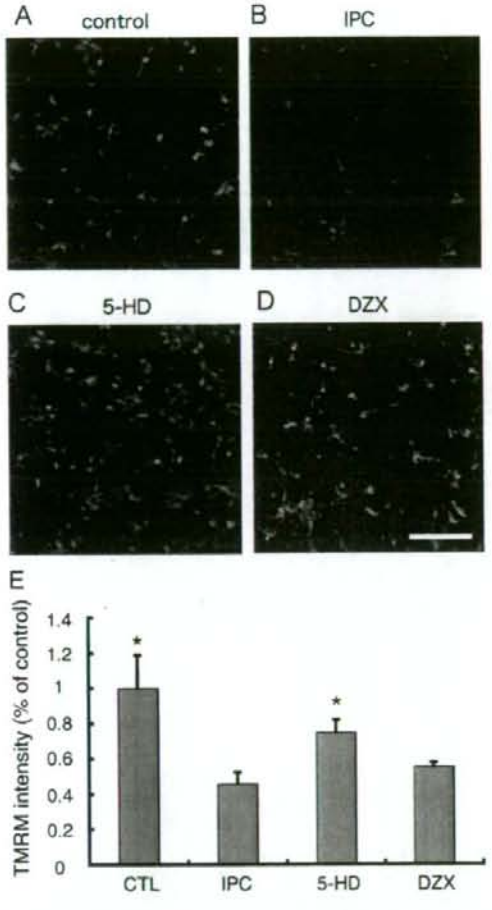

Fig. 4 – TMRM intensities were measured after OGD (panel E). When cells were exposed to short-term OGD (preconditioning) before the lethal OGD, the TMRM intensities were significantly reduced ($p<0.01$, IPC). When cells were pre-exposed with 5-HD before the lethal OGD, the reduction of TMRM intensities was significantly attenuated ($p<0.01$, 5HD). DZX pre-treatment did not affect TMRM intensities (DZX). Upper panels (A–D) show typical TMRM intensity images. *: $p<0.05$ against values of IPC group. Scale bar in panel D shows 100 μm.

medium, extra reduction of TMRM intensities were not observed.

3. Discussion

In this study, we provided results showing that the protective mechanisms observed in cerebral IPC involved the activation of mitoK$_{ATP}$ channel, using three different ischemic tolerance models. Furthermore, we confirmed that ischemic preconditioning induced mitochondrial depolarization and a selective mitoK$_{ATP}$ channel blocker cancelled the depolarization, suggesting that mitochondrial depolarization after mitoK$_{ATP}$ channel activation may be a key phenomenon in cell protection (Sack, 2006).

3.1. *Gerbil tolerance model*

There are a few reports that have studied the relationship between the protective mechanisms of ischemic tolerance and K$_{ATP}$ channels in a gerbil model (Heurteaux et al. 1995, Sorimachi and Nowak 2004) and the results are controversial. Sorimachi and Novak stated that adenosine A1 receptor or K$_{ATP}$ channel activation was not involved in the development of ischemic tolerance; however, they administered glibenclamide (blocker to membrane K$_{ATP}$ channel and mitoK$_{ATP}$ channel) or DPCPX (selective adenosine A1 blocker) before the preconditioning (priming) ischemia (Sorimachi and Nowak, 2004). In our previous study, we administered DPCPX after the first, priming insult (Hiraide et al. 2001), and showed inhibition of the development of protective mechanisms. In the present study, we used selective K$_{ATP}$ channel blocker 5-HD instead of glibenclamide. We understand that Sorimachi and Novak did not rule out the participation of mitoK$_{ATP}$ channel contribution in ischemic tolerance phenomenon.

3.2. *Focal ischemic model*

There is only one report showing a relationship between focal cerebral ischemic tolerance and mitoK$_{ATP}$ channel (Yoshida et al., 2004). In the study, the authors showed that 5-HD administered after the conditioning ischemia cancelled the protective effect of ischemic tolerance, suggesting the involvement of mitoK$_{ATP}$ channel activation. Our results are consistent with those reported results; however, they administered 5-HD at 30 min before 2 h lethal ischemia and we administered 5-HD at 24 h before the lethal ischemia to reduce the direct effect of 5-HD itself. Furthermore, we studied the effect of diazoxide, the selective mitoK$_{ATP}$ channel opener, and showed extra reduction in infarct volumes. Our data more strongly suggest the involvement of mitoK$_{ATP}$ channel activation in focal ischemic tolerance phenomenon.

3.3. *In vitro oxygen–glucose deprivation model with primary cultured neurons*

Figure 3 showed that mitoK$_{ATP}$ is of critical importance in IPC protection of neurons, as shown in the literatures (Reshef et al., 1998, 2000; Kis et al., 2003). However, it remains to be assessed whether IPC-induced mitochondrial depolarization is affected by the modification of mitoK$_{ATP}$. Horiguchi et al. reported that 3-nitropropionic acid (3-NPA)-induced mitochondrial deporalization was blocked by treatment of 5-HD (Horiguchi et al., 2003). In the present study, we added 5-HD to IPC-treated primary cultured neurons and found that mitochondrial depolarization was also blocked by 5-HD (Fig. 4). These results indicate that mitoK$_{ATP}$ is involved in IPC-induced mitochondrial deporalization and subsequent neuronal protection. On the other hand, we did not find any additive effect of mitoK$_{ATP}$ opener DZX in vitro. We interpreted this

result as that once the channels were opened and membrane depolarized, it would be difficult to open or depolarize furthermore in single cell. These results together with cell surviving study (Fig. 3) suggest that the protective effect of IPC involves membrane depolarization, most likely involving the inhibition of Ca^{2+}overload during subsequent lethal ischemia.

3.4. 5-HD and DZX administration

A few in vivo studies have shown neuroprotective effects of diazoxide against cerebral ischemia and it has been shown that the protective effects of diazoxide are cancelled by 5-HD (Shake et al., 2001, Liu et al., 2002, Rajapakse et al., 2002, Shimizu et al., 2002). However, these reports only showed the protective effect of mitoK_{ATP} channel activation in cerebral ischemia, and did not suggest the involvement of mitoK_{ATP} channel activation in ischemic tolerance phenomenon. Neuroprotection induced by mitochondrial K_{ATP} channel opening is possibly explained by optimizing the energy state and suppression of mitochondrial Ca^{2+}overload during ischemia (Shimizu et al., 2002). Diazoxide is suggested to induce mild oxidative stress and preconditioning-like neuroprotection (Samavati et al., 2002). There is also a report showing that 5-HD inhibits the preconditioning effects of diazoxide, while 5-HD given alone produces neuroprotection through suppression of oxidative responses to ischemia (Liu et al., 2003).

To avoid direct toxic effects of 5-HD, we performed the following preliminary experiments. In the in vivo pre-experiments with gerbil, we tried sham+5HD+5 min ischemia and the number of surviving neurons was 3.0±0.71, $n=2$, on the other hand, sham+5 min ischemia showed surviving neurons of 4.4±2.7, $n=5$. Since surviving cells were too small to be compared, we applied a sham+5HD+3 min ischemia model. The number of surviving neurons was, sham+3 min:24.8±13.8, $n=5$, versus sham+5HD+3 min:43.3±39.1, $n=5$. There was no significant direct toxic effect of 5HD observed. In addition with rat, sham+5HD+90 min focal ischemia gave rise to the infarct volume of 113.8±18 mm^3 ($n=5$), in contrast to those of sham+90 min group (198.7±4.6 mm^3, $n=5$). Thus, 5HD seemed to have a protective effect as shown in the literature (Liu et al., 2003) for focal ischemia and did not have a direct toxic effect. In the in vitro pre-experiments, we observed following results of surviving neuronal cell count: IPC only, 255.7±106.7, $n=2$; IPC+5HD (10 μM), 113.8±56.3, $n=2$; IPC+5HD (30 μM), 325.7±245.6, $n=2$; IPC+5HD (100 μM), 456±21.8, $n=2$. Although 5HD concentration was increased up to 100 μM, the number of surviving cell did not decrease. We thought that 5HD did not have direct toxic effects on surviving cells, and the optimal concentration for this in vitro study was determined to be 10 μM.

The effect of diazoxide and 5-HD independent to the mitochondrial potassium channel has been also postulated. Diazoxide attenuates succinate dehydrogenase activity and consequently reduces mitochondrial respiration. 5-HD is converted to its coenzyme A derivative (5HD-Co A), which may affect fatty acid beta-oxidation of mitochondrial metabolism (Hanley et al., 2002, Lim et al., 2002). Thus, these effects may account, in part, for the modification of ischemic tolerance by these agents.

3.5. Mitochondrial membrane potential

TMRM equilibrates in cells according to the Nernst equation and provides a numerical estimation of the mitochondrial membrane potential (MMP) (Zhang et al., 2001). We could observe the depolarization across the mitochondrial inner membrane after ischemic preconditioning and 5-HD administration inhibited the depolarization, as shown in Fig. 4. The mitochondrial membrane depolarization induced by a K^+ influx through mitoK_{ATP} channels is expected to dissipate $\Delta\psi_m$ and thereby decrease Ca^{2+} influx during ischemia. Thus, the driving force for the Ca^{2+} influx through Ca^{2+} uniports into the matrix of mitochondria is reduced at the time of ischemia, consequently attenuating mitochondrial Ca^{2+} overload. Mitochondrial Ca^{2+} overload has been closely related with mitochondrial damage in both necrotic and apoptotic forms of cell death (Siesjö et al., 1995, Halestrap 2006).

In conclusion, we have provided further evidence that the activation of mitoK_{ATP} is involved in development of cerebral ischemic tolerance, probably through mitochondrial depolarization. Signal transduction pathways relating to these events may include various pathways, including protein kinase C, nuclear factor kappaB, nitric oxide synthase, and so on (Centeno et al., 1999, Pérez-Pinzón and Born, 1999, Kirino, 2002, Dirnagl et al., 2003, Sack, 2006). The present findings may help to construct therapeutic strategies for cerebral protection against ischemic damage.

4. Experimental procedures

4.1. In vivo experiment

4.1.1. Transient global ischemia

Male Mongolian gerbils (Meriones unguiculatus, Hoshino Shiken Doubutsu, Saitama, Japan, weighing 60–80 g) were used. Animals were subjected to global ischemia according to a protocol approved by the Animal Care and Use Committee, Nippon medical school, as previously described (Hiraide et al., 2001). Animals were anesthetized with 2% halothane in 30% O_2 and 70% N_2O. Common carotid arteries were isolated and occluded with aneurysm clips. Rectal and temporal muscles were maintained 37–38 °C during ischemia and first 30 min after the ischemia using a heating pad and heating lamp. Then clips were removed to restore blood flow.

At first, 2 min of ischemia were induced, as a sublethal, preconditioning ischemia, then 5 min of ischemia were induced at 24 h later as a lethal ischemia. Thirty minutes before the lethal ischemia, 5-hydroxydecanoate (5-HD) (Sigma H135) 20 mg/kg, diazoxide (DZX) (Wako047-23133) 0.3 mg/kg, or vehicle was administered intraperitoneally. Seven days after the last ischemia, brains were perfusion fixed with 4% formaldehyde in 0.1 mol/l phosphate buffer (pH 7.4) under halothane anesthesia. The slice containing both dorsal hippocampi was stained with hematoxylin eosin (HE). Both sides of intact neurons in the hippocampal CA1 sector were counted and the average of both side counts was calculated and used for the statistics. Hippocampal CA1 neuronal cells were enumerated by an examiner who was blinded to the experimental protocol.

4.1.2. *Focal cerebral ischemia*

Male Sprague Dawley rats (250–300 g) were used. Rats were anesthetized in the same manner as gerbils. The tail artery was cannulated for monitoring physiological parameters (mean blood pressure, blood sugar, and blood gases). Focal ischemia was produced by intraluminal occlusion of the left middle cerebral artery (MCA) with a nylon monofilament with a rounded tip and a distal silicon rubber cylinder. At first, 15 min of ischemia was induced as a sublethal, preconditioning ischemia, then 90 min of ischemia was induced at 48 h later as a lethal ischemia. Twenty-four hours before the lethal ischemia, 20 mg/kg of 5-HD, 0.3 mg/kg of DZX, or vehicle was administered intraperitoneally. At 24 h after MCA occlusion, brains were removed and sliced into 6 coronal sections (2 mm thick). Sections were stained with 2,3,5-triphenyltetrazolium (TTC) (3%), which is a substrate for mitochondrial respiration. The border between infarct and non-infarct tissues was outlined using an image analysis system, and the area of infarction was estimated by subtracting the non-lesioned area of the ipsilateral hemisphere from that of the contralateral side (Swanson et al., 1990). The infarcted area was determined using an image analysis system, and was measured by subtracting the area of non-lesioned ipsilateral hemisphere from that of the contralateral side. The volume of infarction was calculated by integration of the lesioned areas.

4.1.3. *Neurological scoring*

At 24 h after ischemia, a neurological examination was performed. Neurological deficits were scored on a scale of 0–5 as follows (Murakami et al., 1998): 0, no neurological deficit; 1, failure to fully extend the right forepaw; 2, circling to the right; 3, falling to the right; 4, unable to walk spontaneously; and 5, dead. The symptoms just in between the score were evaluated as a half point.

After the scoring of neurological deficit score, Rotameric assessment was performed to test balance and coordination using a rotameric device (Columbus Instruments Rotamex 4/8 system, Ohio, USA). The Rotamex was comprised of a drum that rotated at a decided speed. Rats were placed individually on the rotating drum. Once they were balanced, the drum was switched on at a decided speed. The time in seconds at which each animal fell from the drum was recorded automatically. The average reading of three successive trials was taken from each animal. The test was performed at before ischemia, and at 24 h after ischemia. The results were expressed as percent of before ischemic values.

Then, forelimb grip strength was determined using a grip strength meter (Columbus Instruments, Ohio, USA). The animals were placed on the electronic digital force gauge that measured the peak force exerted by the action of the animal. While being drawn along a straight line leading away from the sensor, the animal released at some point and the maximum force attained was stored on the display. The highest reading of three successive trials was taken for each animal.

4.2. *In vitro experiment*

4.2.1. *Cell culture*

Primary cultured cortical neurons were prepared from 16-day-old rat embryos by a method described previously with modification (Brewer 1995, Furuichi et al., 2005). In brief, neocortical tissues were cleaned of meninges, minced, and treated with protease cocktail (SUMILON). After mechanical dissociation by pipetting, we resuspended cells in nerve-cell culture medium (SUMILON), and then plated the cells on poly-L-lysine-coated 24-well plates at a density of 05×10^4 cells/cm^2 and changed the medium to Neurobasal medium (Invitrogen) with B-27 (Invitrogen) containing 2 mM glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin once every three days (growth medium). At 9 days in vitro (DIV), the growth medium was changed to Neurobasal medium supplemented with B27 minus antioxidant (Invitrogen). At 10 to 12 DIV the cultured cells were used for our experiments. We confirmed neuronal identity by immunostaining with antibodies to neuron marker TUJ-1, and astrocyte marker GFAP and then used preparations only containing over 90% neurons for experiments at 10 to 12 DIV.

4.2.2. *Oxygen–glucose deprivation (OGD) models*

To mimic ischemic conditions in vitro, cells were exposed to OGD as described previously with modification (Goldberg and Choi, 1993, Furuichi et al., 2005). The culture medium was removed and stocked. Cells were gently washed twice with phosphate-buffered saline (PBS), and then placed in OGD medium (Dulbecco's modified Eagle medium [DMEM] without glucose, gassed with 95% N_2/5% CO_2 for 15 min). Cells were incubated at 37 °C for the desired time with a humidified hypoxia chamber gassed with 95% N_2/5% CO_2. OGD was terminated by removing cells from the hypoxic chamber, followed by changing back to the stocked culture medium and further incubation at 37 °C with 95% air/5% CO_2.

4.2.3. *Experimental design*

The cultured neurons were subjected to various OGD treatments, and 24 h after these treatments, viable neurons with a triangular soma and neurites were enumerated under a phase-contrast microscope (×200) in five fields per well for four independent wells. Furthermore, viability in each well was estimated by a modified MTT (3-(4, 5-Dimethyl thial-2-yl)-2, 5-DiphenyltetrazaliumBromide) viability assay according to a Cell Counting Kit (WST-1 assay) (Dojindo). Experimental groups are following: 1) Control group: Cells were incubated with Neurobasal medium supplemented with B27 minus antioxidant in a CO_2 incubator with air, 2) OGD group: Cells were exposed to around 70-min OGD, 3) ischemic preconditioning (IPC)+OGD group: Cells were subjected to IPC (around 8-min OGD) and recovered to normal oxygen-glucose medium. At 24 h after, cells were subjected to around 70-min OGD. 4) IPC+5HD or DZX+OGD group: Cells were subjected to IPC (around 8-min OGD), and recovered. At 24 h, cells were subjected to around 70-min OGD. Thirty min before the OGD, 10 μM 5HD or 10 μM DZX was added to the medium.

4.2.4. *Analysis of mitochondrial membrane potential*

We monitored $\Delta\psi$m (mitochondrial membrane potential) using the $\Delta\psi$m-sensitive dye, tetramethylrhodamine methyl ester (TMRM) (Invitrogen). Neuronal cultures were incubated with 10 nM TMRM in neurobasal medium for 15 min and washed 3 times with PBS. We took fluorescent images with a laser-scanning confocal microscope (Olympus FV300) using

excitation and emission filters of 488 nm and 510 nm, respectively. At that time, fields of views were randomly selected under differential interference contrast optics and the average pixel intensity in individual cell bodies was determined with the use of a computerized image analysis system (Image J).

4.2.5. *Experimental design*

1) IPC group: Cells were subjected to IPC (10-min OGD). Twenty-four hours after the OGD, TMRM fluorescence was monitored. 2) IPC+5HD or DZX+OGD group: Cells were subjected to IPC (around 8-min OGD). Twenty-four hours after the OGD, TMRM fluorescence was monitored. Thirty min before incubation with TMRM, 10 μM 5HD or 10 μM DZX were added to the medium.

4.2.6. *Statistical analysis*

The results are expressed as mean ± SD. To evaluate the difference among groups, one factor analysis of variance (ANOVA) was performed. If there was a significant difference among groups, multiple comparisons were performed with Student–Neuman–Keul's test. For comparing neurological deficit score, Kruskal–Wallis test was performed. If there was a significant difference among groups, multiple comparisons were performed with Steel–Dwass's method.

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Consumption of hydrogen water prevents atherosclerosis in apolipoprotein E knockout mice

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abstract

Oxidative stress is implicated in atherogenesis; however most clinical trials with dietary antioxidants failed to show marked success in preventing atherosclerotic diseases. We have found that hydrogen (dihydrogen; H_2) acts as an effective antioxidant to reduce oxidative stress [I. Ohsawa, M. Ishikawa, K. Takahashi, M. Watanabe, K. Nishimaki, K. Yamagata, K. Katsura, Y. Katayama, S. Asoh, S. Ohta, Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals, Nat. Med. 13 (2007) 688–694]. Here, we investigated whether drinking H_2-dissolved water at a saturated level (H_2-water) ad libitum prevents arteriosclerosis using an apolipoprotein E knockout mouse (apoE $^{-/-}$), a model of the spontaneous development of atherosclerosis. ApoE $^{-/-}$ mice drank H_2-water ad libitum from 2 to 6 month old throughout the whole period. Atherosclerotic lesions were significantly reduced by ad libitum drinking of H_2-water ($p = 0.0069$) as judged by Oil-Red-O staining series of sections of aorta. The oxidative stress level of aorta was decreased. Accumulation of macrophages in atherosclerotic lesions was confirmed. Thus, consumption of H_2-dissolved water has the potential to prevent arteriosclerosis.

Atherosclerosis is a multifactorial and long-lasting process, and atherosclerosis and related cardiovascular diseases represent a state of inflammation and heightened oxidative stress characterized by the accumulation of macrophages and oxidized products of low-density lipoprotein in affected blood vessels [1–3]. Oxidation of low-density lipoprotein is considered an early event; however, most clinical trials supplying a single dietary antioxidant have not resulted in great success in preventing atherosclerotic diseases [1,4–7].

We have reported that molecular hydrogen is an efficient antioxidant by gaseous rapid diffusion into tissues and cells [8]. This finding was soon confirmed by several laboratories [9–12]. Moreover, consumption of water with dissolved molecular hydrogen to a saturated level (hydrogen water) prevents stress-induced cognitive decline in mice [13], and the superoxide formation in mice [14]. A clinical trial showed the decrease in modifying low-density lipoprotein by drinking hydrogen water [15].

Here, we show that consumption of hydrogen dissolved in water has the potential to prevent atherosclerosis using apolipoprotein E knockout (apoE $^{-/-}$) mice, which show impaired clearing of plasma lipoproteins and which develop atherosclerosis in a short time [16,17].

Materials and methods

Animals. Apolipoprotein E-deficient mice (apoE $^{-/-}$) were purchased at the age of 2 months from Taconic. The care and treatment of experimental animals were in accordance with institutional guidelines. This study was approved by the Animal Care and Use Committee of Nippon Medical School.

Hydrogen water administration. Molecular hydrogen (H_2) was dissolved in water under high pressure (0.4 MPa) to a supersaturated level using hydrogen water-producing apparatus (ver. 2) produced by Blue Mercury Inc. (Tokyo, Japan). The saturated hydrogen water was stored in an aluminum bag. Hydrogen water was freshly prepared every week, which ensured that a concentration of more than 0.6 mM was maintained. We confirmed the hydrogen content with a hydrogen electrode (ABLE). Each day, hydrogen water from the aluminum bag was placed in a closed glass vessel (70 mL) equipped with an outlet line containing two ball bearings, which kept the water from being degassed. This vessel ensured that the hydrogen concentration was more than 0.4 mM after one day. Hydrogen water degassed by gentle stirring was used for control

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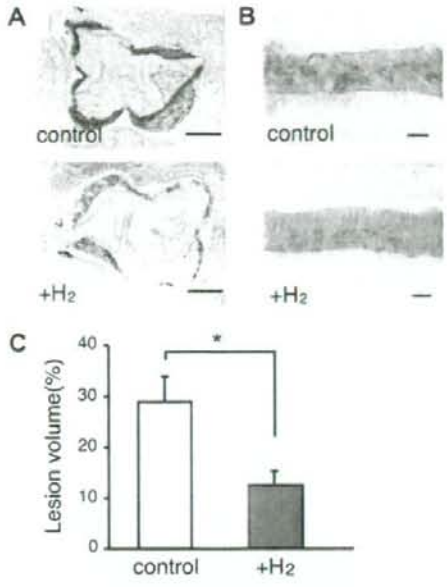

Fig. 1. Consumption of hydrogen water decreased atherosclerotic lesion. ApoE $^{-/-}$ mice drank water containing hydrogen ($+H_2$) or degassed water (control) for 6 months from the age of 2 months old. Representative microscopic pictures of horizontal sections of the proximal aorta attached to the heart (A) and vertical sections of the distal aorta (2 mm from the heart) (B) are shown by Oil-Red-O staining. Scale bar: 100 l m (for A) and 1 mm (for B). (C) Lesion volume was estimated by Oil-Red-O staining of a series of 30 sections (mean value ± SEM, n = 10, p = 0.0069).

animals; the complete removal of hydrogen gas was confirmed with a hydrogen electrode.

Quantification of atherosclerotic lesions in the aorta. The proximal aorta attached to the heart was used to prepare cross-sections. After fixation with 4% paraformaldehyde, cryosections (8 l m) were cut from the site where the aorta valve cups appear at the aorta root. All other sections were collected and stained with Oil-Red-O [18]. The volume of stained lipid (%) was calculated from eight sections for each mouse. The distal aorta (2 mm from the heart) was fixed with 4% paraformaldehyde, opened longitudinally using microscissors and stained with Oil-Red-O.

Immunocytochemistry. After fixation of the proximal aorta with 4% paraformaldehyde, cross-sections (6 l m) were cut with a cryostat, incubated with either an antibody against mouse macrophage (MOMA-2, AbD Serotec), anti-iNOS (BIOMOL), and anti-4-hydroxyl-2-nonenal (HNE) antibody (JaICA, Japan) [19–21]. After washing, the sections were then exposed to a biotinylated second antibody and avidin–peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories Inc.). Sections were developed with DAB as a substrate. One section from each mouse was stained with hematoxylin and eosin (HE).

Statistical analysis. We performed statistical analysis using StatView software (SAS Institute) by applying an unpaired two-tailed Student's t-test and ANOVA followed by Fisher's exact test.

Results

It is easy to consume molecular hydrogen by drinking water containing dissolved molecular hydrogen (hydrogen water). Thus, we examined whether consumption of hydrogen water prevents atherosclerosis using apoE $^{-/-}$ mice. Mice drank nearly the same volume of hydrogen water as control water [4.3 ml/day/mouse(0.1(SD)(hydrogengroup) vs. 4.0 ml/day/mouse(0.1(SD)(controlgroup)]. The amount of food eaten per mouse was also the same in both groups [3.56 ± 0.3 g/day (hydrogen group) vs. 3.28 ± 0.6 g/day (control group)]. After 6 months, we removed the aorta to stain with Oil-Red-O staining. As expected, atherosclerotic lesions were found in 6-month-old apoE $^{-/-}$ mice. In contrast, in mice that had drunk hydrogen water, the volume

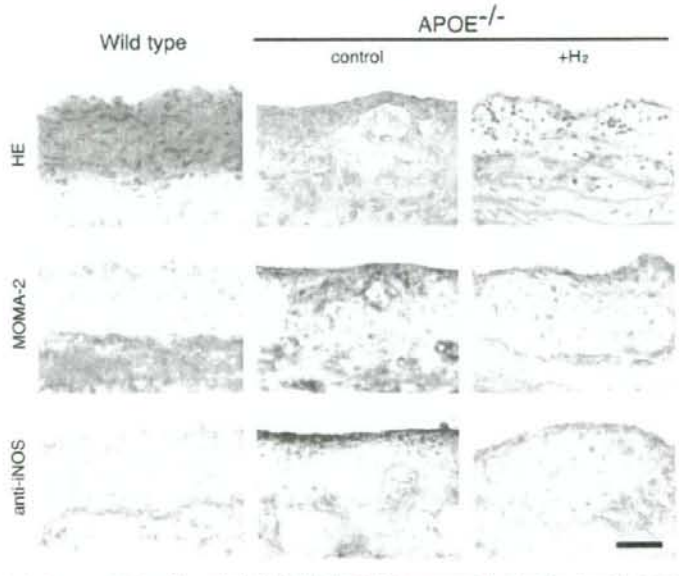

Fig. 2. Representative histochemical or immunostaining of the aorta. ApoE $^{-/-}$ mice drank hydrogen or control water throughout the 6-month period from 2 months old. The proximal aorta attached to the heart was sectioned and stained with HE staining, anti-MOMA-2 immunostaining and anti-iNOS immunostaining. Scale bar: 250 l m.

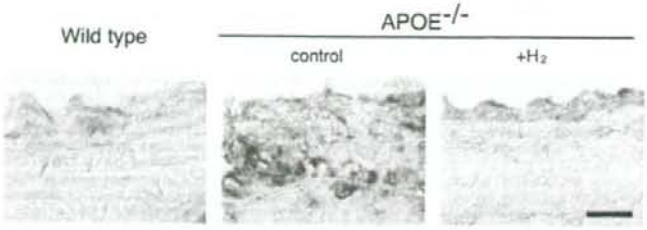

Fig. 3. Representative immunostaining of the aorta. ApoE $^{-/-}$ mice drank hydrogen or control water throughout the 6-month period from 2 months old. The proximal aorta attached to the heart was sectioned and stained with anti-HNE immunostaining. Scale bar: 250 lm.

of atherosclerotic lesion was significantly reduced (Fig. 1). We confirmed that the lesion was derived from macrophage accumulation by staining the sections with anti-MOMA-2 and iNOS antibodies, both of which are macrophage markers [19,20]. (Fig. 2). Moreover, to evaluate the oxidative stress level, we stained the sections with anti-HNE antibody [21]: HNE is an oxidative stress marker (Fig. 3).

These findings suggest that continued consumption of hydrogen water decreased the oxidative stress level and prevented the formation of atherosclerosis, at least in model mice.

Discussion

Clinical evidence as well as experimental results strongly suggests the major contribution of oxidative stress to atherogenesis [1–3]. Thus, dietary consumption of an efficient antioxidant is believed to prevent atherosclerosis; however, the trials have not resulted in great success [1,4–7,22]. Moreover, recent studies have suggested that excessive antioxidant increased the mortality and rates of cancer, because it may interfere with essential defensive mechanisms [23–25]. This may be because low levels of ROS, such as superoxide anion and hydrogen peroxide, function as signaling molecules to regulate apoptosis, cell proliferation, and differentiation [26,27]. The strategy of combining different compounds improved to oxidative status to enable dose reduction of each compound to below the threshold of its side effects [28].

We have found that molecular hydrogen selectively reduces hydroxyl radicals, but not superoxides and hydrogen peroxides that play physiological roles [8]; thus, we suggest that the side effects of hydrogen must be small, different from other antioxidants. Inhalation of hydrogen gas does not influence physiological parameters such as body temperature, blood pressure, pH, and pO_2 in the blood, as shown previously [8,10]. Hydrogen has already been used for humans to prevent decompression sickness in divers at the level of 2 MPa partial pressure of hydrogen, suggesting that 16 mM hydrogen in blood could be safe [29]. When hydrogen water was placed in the stomach, hydrogen was detected in the blood, indicating the incorporation of hydrogen into the body by drinking [13]. Hydrogen diffuses very rapidly into cells, and high efficacy is expected [8,10].

When the preventive level of atherosclerotic lesions in this study is compared with the previous data that apoE $^{-/-}$ mice was used, the efficacy of hydrogen water seems to be greater than folic acid [30], vitamin E [31], iron [32], and a-lipoacid [33]. It is easy to drink hydrogen water daily. We propose that regular consumption of molecular hydrogen dissolved in water has the potential to prevent atherosclerosis. This is the first report that hydrogen water suggests to prevent a lifestyle-related disease. Clinical tests will be needed to elucidate the relevance of hydrogen water to prevent atherosclerosis.

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Clinical Study

Prognosis of glioma patients by combined immunostaining for survivin, Ki-67 and epidermal growth factor receptor

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Abstract

To confirm that survivin expression is a sensitive marker for the prognosis of glioma patients, surgically resected tissues of ninety-nine Japanese glioma patients with antibodies against survivin, Ki-67 and epidermal growth factor receptor (EGFR) were examined immunohistochemically. The median survival of patients with high survivin expression was significantly shorter than that with low expression (322 vs. 1084 days). Ki-67 expression was significantly associated with that of survivin, whereas no significant association between survivin and EGFR expressions was observed. Patients with low EGFR and low survivin expression survived longer than patients with high EGFR and low survivin expression (1509 vs. 795 days). These results indicate that survivin is a highly sensitive marker for glioma prognosis and suggest that the expression levels of survivin and other markers combined, including EGFR, might be a potent tool for the clinical prognosis of glioma patients.

Keywords: EGFR index; Glioma; Immunohistochemical staining; Prognosis; Survivin index

1. Introduction

Glioma, the most common neoplasm in the human brain, includes astrocytoma, anaplastic astrocytoma and glioblastoma multiforme. A significant number of patients rapidly develop malignant glioma.[1] Gliomas are histologically classified into four grades (grades I–IV), according to the World Health Organization (WHO) guidelines. Grade IV glioma has the worst prognosis even after surgical resection, radiation therapy, and chemotherapy. The 5-year survival rate of each grade in Japan is: 66.5% in low-grade astrocytoma (grades I and II), 23.4% in anaplastic astrocytoma (grade III), and 7.0% in glioblastoma (grade IV).[2] However, the outcome of patients in each grade is highly variable[3,4] and genetic differences among them may contribute to their different survival.[5]

Proliferation indexes have been studied to predict the prognostic subgroups of glioma patients. These indexes, including the expression levels of proliferating cell nuclear antigen (PCNA) and Ki-67, are associated with the growth rate of tumor cells when the tumor tissues are surgically removed.[6] The immunohistochemical determination of proliferative activity with the monoclonal antibody MIB-1 against Ki-67, a nuclear antigen, is clinically useful in distinguishing the biological behavior of many tumors. However, there have been conflicting results from using Ki-67 as a prognostic marker for glioma, especially for

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the lower grades.[6] The expression of survivin, a member of the inhibitor-of-apoptosis protein (IAP) family, predicts survival in glioma.[7–9] Survivin is implicated in apoptosis inhibition as well as mitosis regulation.[10,11] Our study suggests that survivin is more sensitive than Ki-67 in helping predict survival for patients with lower grades of glioma.[7]

To clarify the underlying biology and to refine survival prognosis, ancillary studies of several glioma-associated genes and their products have been used.[12,13] Amplification of the epidermal growth factor receptor (EGFR) gene is one of the most frequent alterations to glioma-associated genes, resulting in overexpression of a transmembrane tyrosine kinase receptor.[14] However, results on the prognostic value of EGFR amplification/EGFR overexpression in glioma are inconclusive or inconsistent. Differences in the studied populations may explain this.[15] A region-limited and age-limited study of a population selected from patients with supratentorial glioblastoma multiforme indicated that EGFR amplification/EGFR overexpression was a significant predictor of survival.[16]

To further examine the prognosis of glioma patients, we considered both the current growth status and the genetic background of the tumor. This study shows that the expression levels of survivin and EGFR will be a useful predictor of glioma patient survival. In our retrospective study, we confirmed that survivin was a sensitive marker. In addition we found that the expression level of EGFR in patients with a lower expression level of survivin is associated with their prognosis.

2. Materials and methods

2.1. Clinical data and tissue processing

Ninety-nine Japanese patients with glioma, whose detailed clinical and pathological information was available, were selected from the main and Musashikosugi Hospital of Nippon Medical School (Tokyo and Kawasaki, Japan) from 1996 to 2005. All lesions were classified as primary glioma because the onset of disease was less than three months before diagnosis and there was no prior history of malignant astrocytoma. Patient characteristics, including the Karnofsky performance scale (KPS) score, were collected before initial surgery. Surgical resection, chemotherapy, and radiotherapy were attempted in all patients. Total survival time was defined as the time interval between initial craniotomy and the day of the patient's death. Surgically resected tissues were paraffin-embedded, sectioned at 4 μm thickness, and used for histochemical and immunohistochemical staining. Two or more pathologists and neurosurgeons classified their grades according to WHO guidelines.

2.2. Immunohistochemistry

Anti-survivin antiserum prepared at our Institute was used.[7] Ki-67 and EGFR protein expression was detected using the following monoclonal antibodies: anti-Ki-67 (clone K-2; Ventana, Tucson, AZ, USA) and anti-EGFR (clone 3C6; Ventana). The sections were stained using the Ventana NexES Staining System and all products, except anti-survivin antiserum needed for subsequent steps, were supplied by the manufacturer. Sections were deparaffinized and heated with CC1 cell conditioning solution (denaturing buffer) for 1 hour. After 32 min incubation at 37 °C with anti-survivin antiserum (1:250) or other antibodies, sections were incubated for another 10 min at 37 °C with a secondary biotinylated antibody and then for another 10 min with avidin-peroxidase; 3′,3-diaminobenzidine was used as a chromogen. Slides were counterstained in Mayer hematoxylin, dehydrated, and mounted.

2.3. Evaluation of the results of immunohistochemical staining

Stained sections were observed under a microscope. Survivin, Ki-67, and EGFR indexes were determined as the percentage of immunostained cells per 200 cells by 5 fields of view in each section. The survivin index was classified as 'low' if <50% of cells were stained, and 'high' if >50% of cells were stained. The Ki-67 index was 'low' if 0%–10% of cells were stained and 'high' if >10% of cells were stained. The EGFR index was 'low' <50% of cells were stained and 'high' if >50% of cells were stained.

2.4. Statistical analysis

Statistical analysis was performed using SPSS software (SPSS, Chicago, IL, USA). Survival was followed for up to 2000 days and the Kaplan–Meier method was used to calculate curves.[17] The survival periods and the strength of associations between categories were compared with the log-rank test (Mantel–Cox) for univariate analysis.[18] Multivariate analysis was performed using the Cox regression model. Fisher's exact test or χ^2 test was used to analyze the distribution of the survivin index according to other variables, and a level of $p < 0.05$ was considered significant.

3. Results

3.1. Patient characteristics

The age of patients ($n = 99$) at the time of surgery ranged from 8 to 82 years; the mean age was 53.8 years, and the median KPS score was 70% (range, 50%–100%). The patients were 55 men and 44 women diagnosed with low-grade astrocytoma ($n = 18$), anaplastic astrocytoma ($n = 34$) and glioblastoma ($n = 47$) (Table 1). Seventy-eight of the ninety-nine patients died during follow-up. The median survival was 383 days after initial craniotomy (55 weeks; range 5–286 weeks). We found different expression profiles for survivin, Ki-67 and EGFR in each grade of glioma (Table 1).

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Table 1
Patient demographics

Histological diagnosis and grade	No. patients	Mean age (yrs)	Gender		Median survival (days)	Survivin		Ki-67		EGFR	
			Male	Female		High	Low	High	Low	High	Low
Grade II	18	47.4	9	9	1235 (238–2000)	4	14	1	17	1	17
Anaplastic astrocytoma (Grade III)	34	53.9	20	14	358 (30–1628)	23	11	22	12	9	25
Glioblastoma (Grade IV)	47	56.0	26	21	325 (36–2000)	36	11	35	12	15	32
Total	99	53.8	55	44	383 (30–2000)	63	36	58	41	25	74

EGFR = epidermal growth factor receptor.

Table 2
Univariate analyses with the Cox–Mantel log-rank test of the effect on median survival

Variable		No. of patients	Median survival (days)	
Age	⩾55	54	303	$p = 0.0102$
	<55	45	629	
Gender	Male	55	363	$p = 0.1400$
	Female	44	421	
KPS	>70	45	819	$p < 0.0001$
	⩽70	54	267	
Survivin	High	63	322	$p < 0.0001$
	Low	36	1084	
Ki-67	High	58	312	$p < 0.0001$
	Low	41	795	
EGFR	High	25	312	$p = 0.0800$
	Low	77	404	
Grade	II	18	1235	$p = 0.0002$
	III	34	358	
	IV	47	325	

EGFR = epidermal growth factor receptor, KPS = Karnofsky performance scale.

3.2. Significant prognostic value of survivin index for glioma

Univariate analyses of each factor with Mantel–Cox log-rank analysis (Table 2) show that age, KPS score, grade, survivin index and Ki-67 index were significantly associated with prognosis. Among them, the KPS score, survivin index and Ki-67 index were the most significant ($p < 0.0001$). Almost all sections from glioma patients in various WHO grades were stained immunopositive with anti-survivin antiserum (Fig. 1A–C), whereas normal brain tissue was not (Fig. 1D). Our antiserum detected both cytoplasmic and nuclear forms of survivin.[7] The distribution patterns of survivin in glioma cells were highly variable and might be due to the phenotype of gliomas.[19] The median survival of patients with a high survivin index was significantly shorter than those with a low survivin index (322 vs. 1084 days, $p < 0.0001$, Table 2, and Fig. 2A), whereas the survivin index varied widely in each grade (Table 1).

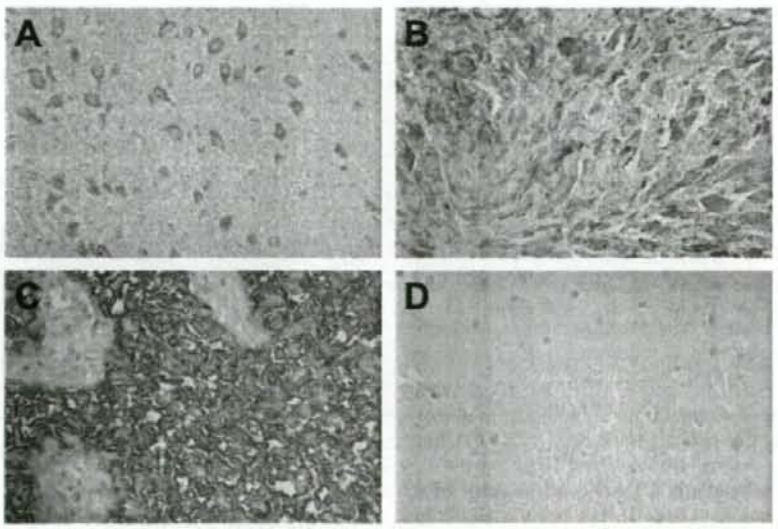

Fig. 1. Immunohistochemical staining of gliomas with anti-survivin antiserum. Surgical sections from patients with different World Health Organization grades of glioma were stained with 1:250-diluted anti-survivin antiserum using the Ventana NexES Staining System. Low-grade astrocytoma (A), anaplastic astrocytoma (B), glioblastoma (C), and normal brain tissue (D). Scale bar: 50 μm.

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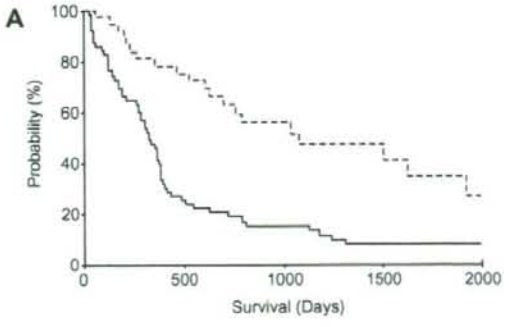

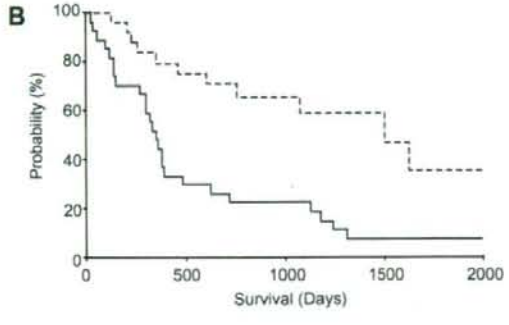

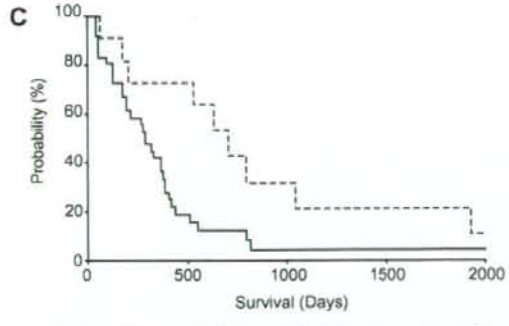

Fig. 2. Kaplan–Meier survival plots defined by the survivin index of patients with grades II–IV (A) or with grades II and III (B), and IV (C). In each grade, glioma patients with low survivin index (dotted lines) survived for significantly longer than patients with high a survivin index (solid lines).

The Kaplan–Meier survival curves (Fig. 2B, C) showed that the survivin index was not only a significant predictor of survival in high-grade glioma (grade IV, $p = 0.0207$), but also in lower-grade glioma (grades II and III, $p = 0.0004$). The Ki-67 index was similarly a significant predictor of a lower grade of glioma ($p = 0.0002$), but not a high-grade glioma ($p = 0.2681$). In multivariate analysis, the KPS score, grade and survivin index were significant predictors of survival (Table 3). These results are consistent with our preliminary work and suggest that the survivin index, which is inversely correlated with the survival of lower-grade gliomas, will be a powerful tool for clinical prognosis.

Table 3
Multivariate analyses with Cox regression model

Variables		Relative risk (95% CI)	P
Age	⩾55	1.225 (0.753 – 1.992)	0.4158
Gender	Female	0.803 (1.045 – 2.713)	0.3816
KPS	>70	0.304 (0.181 – 0.512)	<0.0001
Survivin	High	1.931 (1.079 – 3.456)	<0.0269
Grade	IV	1.684 (1.045 – 2.713)	0.0322

CI = confidence intervals, KPS = Karnofsky performance scale.

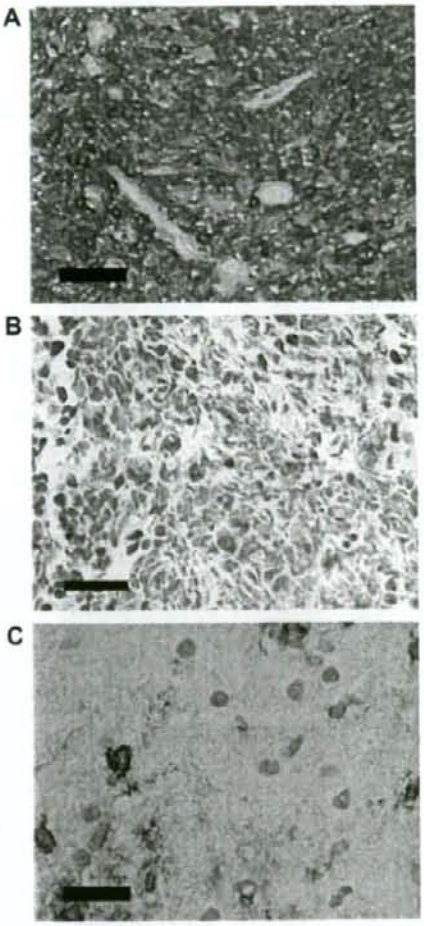

Fig. 3. Immunohistochemical staining of gliomas with anti-epidermal growth factor receptor (EGFR) antibody (clone 3C6). Surgical sections from patients with different World Health Organization grades of glioma were stained with 1:250-diluted anti-EGFR antibody using the Ventana NexES Staining System. A glioblastoma showed a high (A) or low (B) expression of EGFR. A low-grade astrocytoma expressing EGFR is also shown (C). Scale bar: 50 μm.

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3.3. Associations between survivin index and other variables

Fisher's exact test or χ^2 test (Table 4) showed no significant statistical association of the survivin index with gender and EGFR index ($p > 0.05$), indicating that these variables might not affect the expression of survivin. A significant association of the survivin index with age, KPS score and grade was observed ($p = 0.0017$, 0.0006 and 0.0002, respectively). This indicated that older patients with glioblastoma multiforme (grade IV) and a lower KPS score, and patients with a lower KPS score, tend to express a high level of survivin. A significant association of the survivin index with the Ki-67 index was also observed ($p = 0.0002$). The result confirms that both indexes exhibit similar characteristics as potent indicators of survival.

3.4. Combined prognosis with survivin and EGFR indexes

The survivin index correlated strongly with the Ki-67 index, but not with the EGFR index ($p = 0.0573$). This indicated that the EGFR index might reflect the distinct status of tumor progression. Stained sections from glioma patients of various grades with anti-EGFR antibody are shown in Fig. 3. Patients with a high EGFR index exhibited slightly shorter survival (312 vs. 404 days, $p = 0.0800$, Table 2). Thus, the prognostic value of the EGFR

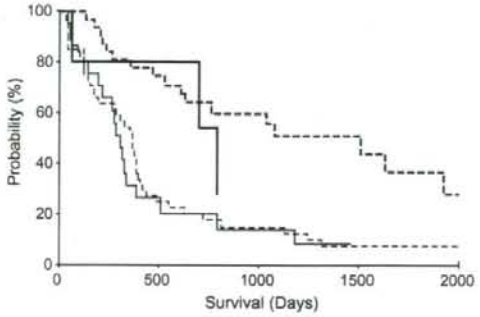

Fig. 4. Kaplan–Meier survival plot of four groups defined by high/low survivin and high/low EGFR indexes. Bold and dotted line, low survivin and low EGFR group; bold and solid line, low survivin and high EGFR group; thin and dotted line, high survivin and low EGFR group; thin and solid line, high survivin and high EGFR group.

Table 4
Survivin expression versus other variables

Variables		Survivin		P
		Low	High	
Age				0.0017
	<55	24 (53)	21 (47)	
	⩾55	12 (22)	42 (78)	
Gender				0.4104
	Male	18 (41)	26 (59)	
	Female	18 (33)	37 (67)	
KPS				0.0006
	>70	20 (44)	25 (56)	
	⩽70	43 (80)	11 (20)	
Ki-67				0.0002
	Low	24 (59)	17 (41)	
	High	12 (21)	46 (79)	
EGFR				0.0573
	Low	31 (42)	43 (58)	
	High	5 (20)	20 (80)	
Grade				0.0002
	II	14 (78)	4 (22)	
	III	11 (32)	23 (68)	
	IV	11 (23)	36 (77)	

Values in parentheses indicate percentage.
EGFR = epidermal growth factor receptor, KPS = Karnofsky performance scale.

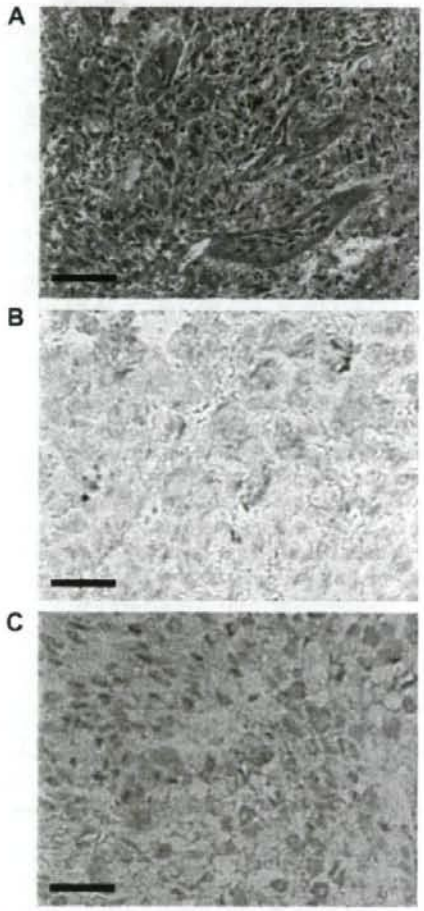

Fig. 5. Histochemical staining of a long-surviving glioblastoma patient. Sections were stained with hematoxylin and eosin (A), anti-survivin antiserum (B) and anti-epidermal growth factor receptor (EGFR) antibody (C). Scale bar: 50 μm.

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index by itself seems low for glioma. Patients with low EGFR and low survivin indexes survived longer than patients with high EGFR and low survivin indexes (1509 vs. 795 days, Fig. 4). However, patients with a high survivin index survived almost the same length of time whether the EGFR indexes were low or high (363 vs. 281 days). Surprisingly, there were eight patients with grade IV glioma among the thirty-two patients with both low survivin and low EGFR indexes (Fig. 5). The median survival of these eight patients was 628 days. This suggests that a prognosis based on combined survivin and EGFR indexes might be a prominent tool for more accurate prognosis of glioma patients.

4. Discussion

Because standard histological methods do not precisely predict which tumors will undergo rapid malignant progression, it is difficult to give an accurate prognosis to patients. Individual markers alone or combined with histological grading generally neither predict survival nor are able to indicate therapeutic treatments. There are too many physiological, biological, and therapeutic differences in glioma patients to predict their survival periods. We found, however, that the immunohistological determination of survivin expression alone was strongly associated with the prognosis of glioma patients (Tables 2 and 3). Many cells in all grades of glioma stained positive for survivin (Table 1, Fig. 1). The survivin index was predictive, especially in lower-grade gliomas (Table 2 and Fig. 2). Previous studies demonstrated that survivin is expressed at G2/M in a cell-cycle-dependent manner[20] and is associated with kinetochores of metaphase chromosomes and the central spindle midzone at anaphase.[21] This suggests that survivin participates as a chromosomal passenger protein in the formation of the cleavage furrow. Forced expression of survivin counteracted cell death induced by various apoptotic stimuli, whereas interference with the expression or function of survivin by a dominant negative form, or antisense of survivin, caused spontaneous apoptosis and multiple cell division defects,[22] suggesting that survivin acts both as a mitotic regulator and as a cytoprotective factor at cell division. We observed the accumulation of survivin both in the nucleus and the cytoplasm (Fig. 1). Nuclear and cytoplasmic survivin may indicate cell proliferation and anti-apoptotic potential, respectively. A higher amount of survivin accumulates in the cytoplasm of more malignant glioma cells.[19] These two different functions of survivin might be suitable for prognosis of the survival period of glioma patients.

Most gliomas express EGFR[23] (Table 1; Fig. 3), indicating that the activation of EGFR might be one of the primary causes of glioma onset. EGFR stimulation with appropriate ligands facilitates the expansion of glioma growth, migration and invasion[24]; but our results showed that the EGFR index was weakly associated with prognosis (Table 2) and did not correlate with the survivin index (Table 4). Several multivariate approaches revealed that EGFR expression and proliferation markers were independent predictors of survival.[25–28] A possible explanation for this discrepancy is that the EGFR index does not reflect the activation level of EGFR. Thus, we suggest that EGFR in glioma with a low survivin index might still be inactive, and would become active to facilitate tumor progression. In the population with a low survivin index (Fig. 4), the significant difference in survival between patients with high and low EGFR indexes supports our hypothesis. The shorter survival of patients with a high EGFR index might be due to the later activation of survivin. Thus, we propose that the determination of activated EGFR and the subsequent signaling pathway will be helpful in evaluating the prognostic value of EGFR and developing anti-EGFR therapies. The EGFR index did not affect the survival of patients with a high survivin index (Fig. 4). The shorter survival of high survivin-index patients with a low EGFR index might be due to other molecular characteristics associated with proliferation (e.g. platelet-derived growth factor receptor, PDGFR; methionine supply, Met) and invasion.[29] Taken together, these results demonstrate that the EGFR index could provide a prognosis of the survival period for patients with glioma who exhibit a low survivin index.

The survivin index itself showed a significant association with prognosis in patients with glioma and, furthermore, the EGFR index might be associated with prognosis in a subset of patients defined by the survivin index. The extremely long survival of patients histologically diagnosed with glioblastoma multiforme without a high expression of both survivin and EGFR (Fig. 5) supports the advantage of combining the immunological diagnosis with the expression levels of survivin and EGFR. Our findings will not only be useful for understanding glioma, but for effective clinical diagnosis. Additional studies about *in vivo* molecular signaling and/or cofactors of EGFR to induce a high expression of survivin in glioma are likely to further highlight the advantage of combinational diagnosis with survivin and EGFR.

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