

# Hypoxia-induced cell death of HepG2 cells involves a necrotic cell death mediated by calpain

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**Abstract** To elucidate mechanism of cell death in response to hypoxia, we attempted to compare hypoxia-induced cell death of HepG2 cells with cisplatin-induced cell death, which has been well characterized as a typical apoptosis. Cell death induced by hypoxia turned out to be different from cisplatin-mediated apoptosis in cell viability and cleavage patterns of caspases. Hypoxia-induced cell death was not associated with the activation of p53 while cisplatin-induced apoptosis is p53 dependent. In order to explain these differences, we tested involvement of  $\mu$ -calpain and m-calpain in hypoxia-induced cell death. Calpains, especially  $\mu$ -calpain, were initially cleaved by hypoxia, but not by cisplatin. Interestingly, the treatment of a calpain inhibitor restored PARP cleavage that was absent during hypoxia, indicating the recovery of activated caspase-3. The inhibition of calpains prevented proteolysis induced by hypoxia. In addition, hypoxia resulted in a necrosis-like morphology while cisplatin induced an apoptotic morphology. The calpain inhibitor prevented necrotic morphology induced by hypoxia and converted partially to apoptotic morphology with nuclear segmentation. Our result suggests that calpains are involved in hypoxia-induced cell death that is likely to be necrotic in nature and the inhibition of calpain switches hypoxia-induced cell death to apoptotic cell death without affecting cell viability.

**Keywords** Apoptosis · Necrosis · Calpain · Hypoxia · HepG2

## Introduction

In most tumors, hypoxia develops as a result of inefficient vascular development. Hypoxic cells comprise clinical problems, because they are more resistant to radiotherapy and to many chemotherapeutic agents than their normoxic counterparts [1]. In addition, hypoxia could produce a more aggressive phenotype and contribute to metastasis [2]. Alternatively, hypoxia may induce the cell death of tumor cells [3]. The hypoxic cell death has been reported to have an apoptotic basis, but it might have some necrotic characteristics [4]. In spite of numerous *in vitro* and *in vivo* studies, the mechanism of cell death induced by hypoxia remains to be elucidated.

Cisplatin is an important chemotherapeutic agent and is used widely for treatment of various tumors [5]. Cisplatin cross-links DNA and the resulting DNA adducts are poorly repaired by nucleotide excision repair system [6]. Although it is still debatable whether clinical success of cisplatin relies primarily on its ability to trigger apoptosis, several mechanisms have been proposed concerning cisplatin-induced apoptosis [7, 8]. The most feasible mechanism of cisplatin-induced cell death involves tumor suppressor protein p53 or its family, p73 [9, 10].

p53 is a transcriptional activator in mammalian cells and is involved in apoptosis and cell cycle arrest. The accumulation of p53 following genotoxic stress involves posttranscriptional regulations such as enhanced translation of p53 mRNA and decreased proteolytic degradation [11]. p53 is also regulated posttranslationally by multiple phosphorylations, dephosphorylations, and acetylations [12, 13]. Although these

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posttranslational modifications are known to increase the sequence-specific DNA binding activity of p53 and its trans-activation properties, the physiological significance remains to be determined.

Hypoxia is a physiological inducer of the p53 tumor suppressor gene product and can apply selective pressure during tumor growth to eliminate cells with wild-type p53, and to promote the clonal expansion of cells with mutant forms or inactive p53 proteins [14]. Similar to other DNA damaging agents that causes p53 stabilization by MDM2 (Mouse Double Min 2), the hypoxic accumulation of p53 may be due to the p53 stabilization by hypoxia inducible factor (HIF)-1 $\alpha$  [15], and cause subsequent nuclear and mitochondrial DNA damage [16].

Caspase cascade has been well documented to play a dominant role in diverse stimuli that trigger apoptosis [17]. Caspases have cysteine protease activity and cleave target proteins, which include adapter and effector caspases. In particular, the activation of final effector caspase-3 causes apoptotic characteristics such as DNA fragmentation and nuclear condensation or segmentation. However, not every apoptotic pathway proceeds in a caspase-dependent manner [18]. Recently, calpains, other types of cysteine proteases, were found to be involved in the apoptosis of many cell types [19, 20]. Calpains are Ca<sup>2+</sup>-dependent cysteine proteinases, and facilitate the apoptotic process by cleaving cytoskeleton proteins. There are two major calpains,  $\mu$ -calpain and m-calpain, and possible interactions between caspases and calpains have been reported [21].

To clarify mechanisms of hypoxia-induced cell death, we attempted to compare hypoxic cell death of HepG2 hepatoblastoma cells containing wild-type p53 with cisplatin-induced apoptosis. The dominant pathway involved in hypoxia-induced cell death and the roles of the molecules in the pathway were investigated. Here we report that hypoxia-induced cell death involves calpains, and that the inhibition of calpain activities causes a change from necrosis-like cell death to apoptotic pathway by resulting in caspase-dependent cell death.

## Materials and methods

### Materials

Cisplatin, Methylthiazolotetrazolium (MTT), and Methylene Blue were purchased from Sigma Chemicals. Calpain inhibitors, N-Acetyl-Lue-Lue-Norleu-al (ALLM) and N-Acetyl-Leu-Leu-Met-al (ALLN), were obtained from Calbiochem. N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk), broad range caspase inhibitor, was obtained from PharMingen. Chemicals for electrophoresis were purchased from Bio-Rad and Fisher Scientific. Other

materials were obtained from Sigma Chemicals. Anti-p53 (DO-1) mouse monoclonal, anti-p27 (F-8) mouse monoclonal, anti-human capase-3 (H-277) rabbit polyclonal, anti-caspase-8 (T-16) goat polyclonal antibodies were purchased from Santa Cruz. Anti-Bax (Ab-4) mouse monoclonal and anti-HIF1 $\alpha$  mouse monoclonal were purchased from Transduction Laboratory. Anti-p21 (HZ52) mouse monoclonal, anti-p16 (16P04) mouse monoclonal and anti-Bcl-xL (7D9) mouse monoclonal were from NeoMarkers. Anti- $\mu$ -calpain (Ab3104) and anti-m-calpain (107–82) were obtained from Chemicon and Biomol, respectively.

### Culture and hypoxia incubation

HepG2, a human hepatoblastoma cell line containing wild type p53, was purchased from the American Type Culture Collection. Cells were cultivated in Dulbeccos Modified Eagles Medium supplemented with 10% fetal bovine serum (Cambrex) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were plated at a confluence of 50–60% in 100-mm dish or 24 multi-well plate and the culture medium was changed 2 h before treatment to assure an adequate supply of nutrients and growth factors. For hypoxia treatment of HepG2, cell culture dishes were placed in an anaerobic chamber (Forma scientific) containing incubator unit filled with a defined gas mixture. For stringent hypoxia experiments, this gas mixture contained 85% N<sub>2</sub>, 10% H<sub>2</sub>, 5% CO<sub>2</sub> and a palladium catalyst that scavenge any contaminating oxygen by combining with hydrogen, bringing the oxygen concentration to < 0.01%. For cisplatin treatment, cisplatin was dissolved in dimethyl sulfoxide (DMSO) and treated at 2  $\mu$ g/ml and 16  $\mu$ g/ml. Control groups were treated with DMSO. Both hypoxia and cisplatin treatments were for up to 72 h and the treated cells were harvested every 24 h. caspase inhibitors or calpain inhibitors were treated for 60 min before hypoxia or cisplatin incubation.

### Western blot analysis

To prepare whole cell lysate, cells were washed twice with ice-cold PBS and resuspended in a 0.5 M Tris-HCl (pH 7.4) buffer containing 150 mM NaCl, 50 mM EDTA, 1% NP40, 4 mM phenylmethylsulfonylfluoride (PMSF), 1  $\mu$ g/ml aprotinin and 1  $\mu$ g/ml leupeptin. The lysed cells were vortexed and centrifuged at 17,000  $\times$  g for 20 min at 4°C. The clarified supernatants were stored at –70°C. Subcellular fractionation was performed to prepare cytosol to measure the localization of cytochrome C. Briefly, cells were washed twice with ice-cold PBS and resuspended in 1 ml of hypotonic lysis buffer containing 10 mM HEPES (pH 7.4), 10 mM NaCl, 1 mM PMSF, 1  $\mu$ g/ml leupeptin and 1  $\mu$ g/ml aprotinin and incubated on ice for 30 min. Cells were then disrupted by 6–7 cycles of freezing in liquid nitrogen and thawing at 37°C.

After trypan blue staining to verify more than 95% of cells are disrupted, the crude lysate was centrifuged at  $100,000 \times g$  for 1 h at  $4^{\circ}\text{C}$ . The resulting supernatant and the whole cell lysate were run on a SDS-PAGE gel after protein concentrations were determined by Lowry assay (Biorad). The resolved proteins were then electroblotted onto a PVDF membrane. After blocking in 5% skim milk for 1 h, the membrane was incubated for 1 h with primary antibodies and then incubated with horseradish peroxidase-conjugated secondary antibodies. The bands were visualized by chemiluminescent substrates according to a manufacturer's procedure (Amersham).

#### DNA fragmentation assay

After harvesting cells, cells were washed twice with ice-cold PBS and resuspended in a lysis buffer containing 10 mM EDTA, 0.5% SDS, 50 mM Tris-HCl (pH 8.0) and 200  $\mu\text{g}/\text{ml}$  proteinase K. The cell suspension was incubated at  $50^{\circ}\text{C}$  for 1 h and then the same volume of 10 mg/ml of RNase A was added. After incubation at  $37^{\circ}\text{C}$  for 30 min, the resultant solution was mixed with 1/4 volume of 1% low-melting agarose and 0.5% Tris-borate-EDTA preheated at  $70^{\circ}\text{C}$ . Then the samples were loaded on dry wells of 2% agarose gel and electrophoresed for 2 h at  $100\text{ V}$  at room temperature. The gel was stained in 2 mg/ml of ethidium bromide solution for 1 h and visualized by UV after destaining in water.

#### MTT reduction test

Cell viability was estimated by measuring the rate of mitochondrial reduction of MTT. Briefly, cells were seeded at  $1 \times 10^5$  cells per well in 100  $\mu\text{l}$  of medium in 96-well plates and MTT was added at 1 mg/ml final concentration. After 1 h of incubation at  $37^{\circ}\text{C}$ , the media was removed and 100  $\mu\text{l}$  of DMSO was added. The absorbance at 570 nm was measured using a microplate reader (Bio-Rad). For protein determination per well, methylene blue was used [22]. Cells in 96-well plates were fixed with 100  $\mu\text{l}$  of 4% formalin for 10 min after washing twice with PBS. 100  $\mu\text{l}$  methylene blue was added and incubated for 10 min. After removing and washing methylene blue, 100  $\mu\text{l}$  of 0.1 N HCl was added and the mixture was incubated for 15 min at room temperature. The plate was subjected for measuring OD at 660 nm.

#### Measurement of cellular DNA content by flow cytometry

After hypoxic incubation,  $1 \times 10^6$  cells were harvested by trypsinization. The collected cells were washed in ice-cold PBS and fixed with 70% ethanol at  $-20^{\circ}\text{C}$ . Cells were incubated in 0.4 mg/ml propidium iodide with PBS containing 0.5% Triton X-100 for 30 min. And then FACS-calibur flow

cytometer (BD bioscience) was used to measure the cellular DNA content with an excitation laser at 488 nm and a 575 nm band pass filter.

#### Electrophoretic mobility shift assay (EMSA)

To prepare nuclear extract, cells were washed twice with ice-cold PBS and resuspended in 1 ml of hypotonic lysis buffer containing 10 mM HEPES (pH 7.4), 10 mM NaCl, 1 mM PMSF, 1  $\mu\text{g}/\text{ml}$  leupeptin and 1  $\mu\text{g}/\text{ml}$  aprotinin and incubated on ice for 30 min. After the incubation, the crude mixture was centrifuged at  $10,000 \times g$  for 1 h at  $4^{\circ}\text{C}$ . Next, resultant pellet was resuspended with same lysis buffer in appropriate volume. The pre-mix of DNA-protein binding reactions contained 20 mM HEPES (pH 7.9), 1 mM DTT, 3.5 mM  $\text{MgCl}_2$ , 100 mM KCl, 0.03% Nonidet P-40, 10% glycerol, 1  $\mu\text{g}$  poly(dI.dC), and 5  $\mu\text{g}$  nuclear extract in 20  $\mu\text{l}$  final volume. Reactions were carried out for 10 min, followed by the addition of 1 ng [ $^{32}\text{P}$ ]-labeled p53-consensus oligonucleotide and incubated further for 20 min at  $22^{\circ}\text{C}$ . The entire reaction mixture was loaded directly on a native 4% polyacrylamide gel. After electrophoresis, the DNA protein complexes were visualized by autoradiography. For competition experiments, a molar excess of unlabeled oligonucleotide was added to the reaction mixture 10 min before the addition of [ $^{32}\text{P}$ ]-labeled.

#### Measurement of *in vitro* PARP cleavage activity

To measure the caspase-3 activity of hypoxic HepG2 cells, we performed *in vitro* PARP cleavage activity. Briefly, we prepared nuclear extract of normal growing HepG2 for western blots of PARP and the cytosol of hypoxic HepG2 at indicated times (0, 24, and 72 h) to detect activated caspase-3 activity. To prepare nuclear extract, HepG2 cells were resuspended in 1 ml of hypotonic lysis buffer containing 10 mM HEPES (pH 7.4), 10 mM NaCl, 1 mM PMSF, and 1  $\mu\text{g}/\text{ml}$  aprotinin and incubated on ice for 30 min. The resultant lysate was centrifuged at  $1,000 \times g$  for 15 min at  $4^{\circ}\text{C}$  and the pellet was resuspended in the same buffer containing 0.5% NP-40. The cytosolic preparation for cytochrome C release was done as above without cysteine protease inhibitors such as leupeptin. Finally, 30  $\mu\text{g}$  of nuclear and cytosolic lysates each were mixed and incubated at  $37^{\circ}\text{C}$  for 2 h. The mixture was analyzed with SDS-PAGE and western blot of PARP.

#### Electron microscopy

HepG2 cells grown in 60-mm culture dishes ( $1-3 \times 10^6$  cells) were gently pelleted by centrifugation and washed twice with PBS. Cells were fixed in 100 mM phosphate buffer, pH 7.4, containing 2.5% glutaraldehyde for 30 min

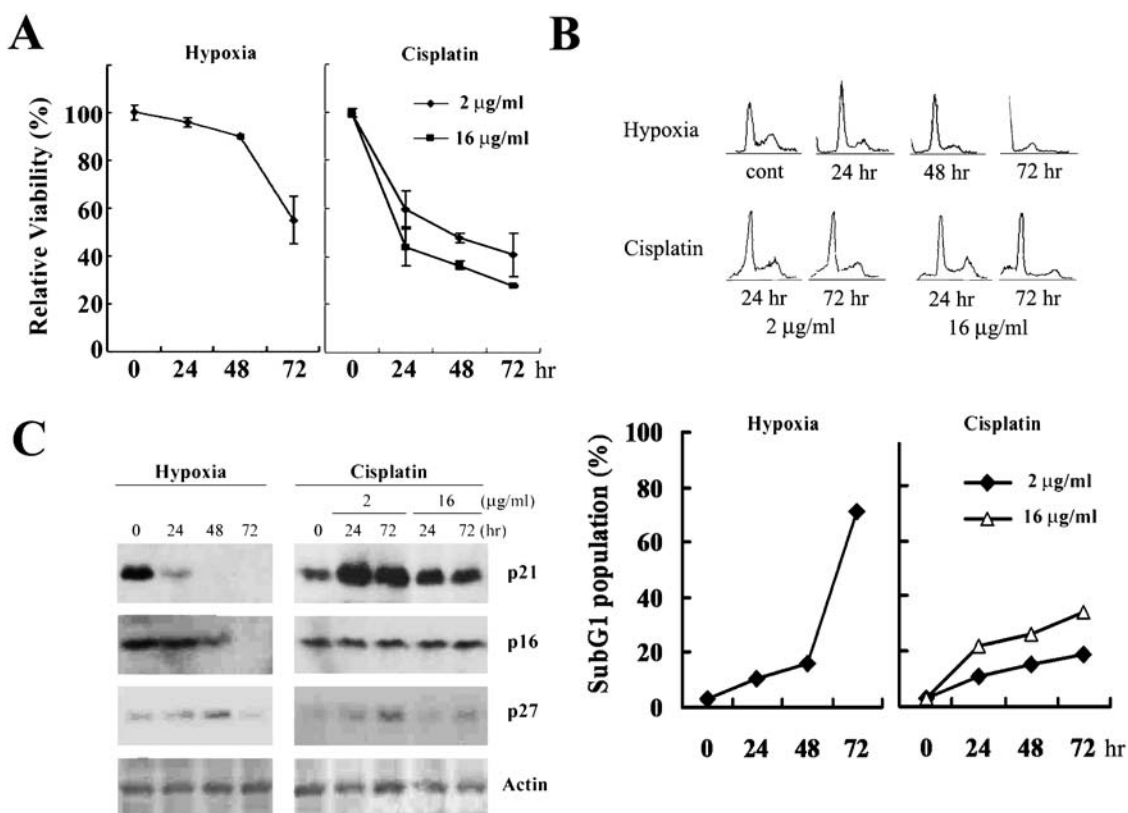
at 4°C. The pellets were rinsed twice with cold PBS, post-fixed in OsO<sub>4</sub>, dehydrated in graded acetone, and embedded in Durcupan ACM resin (Fluka, Switzerland). Ultrathin sections were prepared with Ultra-microtome (Reichert-Jung, Germany), mounted in copper grids, and counterstained with uranyl acetate and lead citrate. Photographs were taken using Zeiss EM 109 electron microscope (Germany).

## Results

### Changes in viability of HepG2 cells during treatment of hypoxia and cisplatin

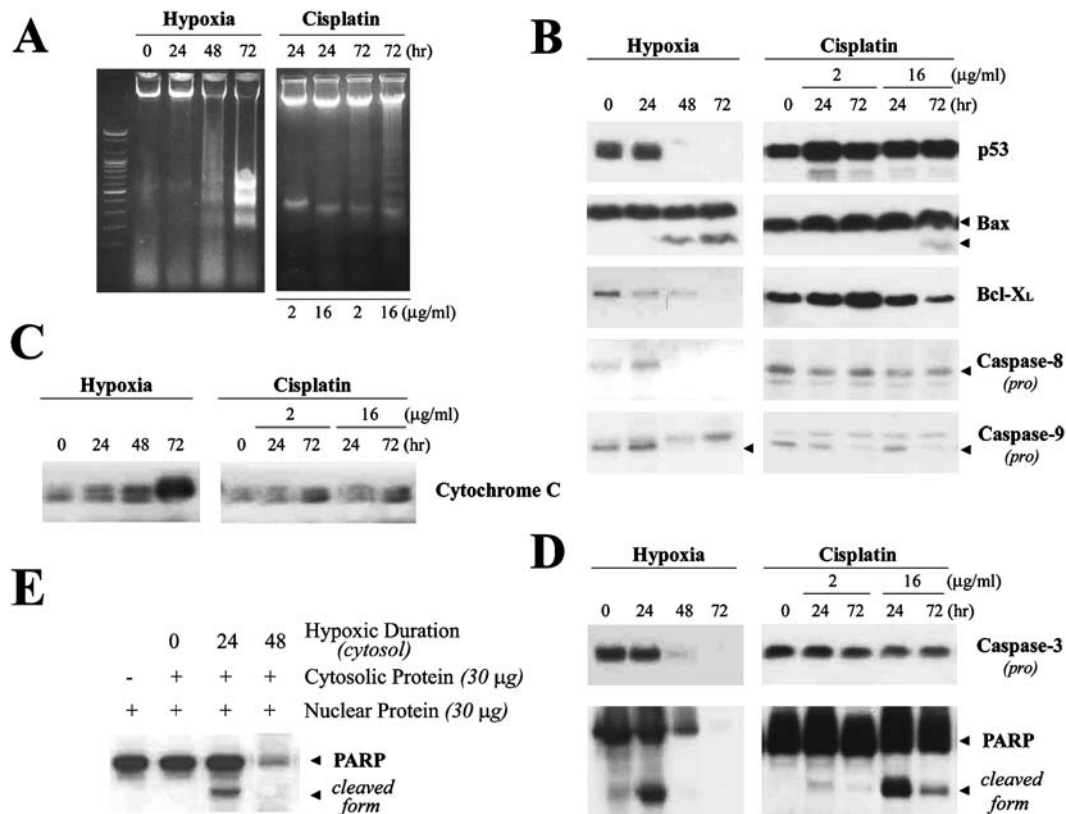
MTT mitochondrial reduction test showed that the cell death of HepG2 was apparent in both hypoxia-induced and cisplatin-treated groups (Fig. 1(A)). The viability of HepG2 cells decreased abruptly in the third day of hypoxia

treatment, while cisplatin-induced cell death varied in a dose and time dependent manner. The subG<sub>1</sub> populations of hypoxic HepG2 cells exhibited the same pattern as shown in the MTT reduction test (Fig. 1(B)). The viability of hypoxic HepG2 cells decreased rapidly at 72 h after hypoxia treatment and G<sub>1</sub> and G<sub>2</sub>/M peaks disappeared. But, the cisplatin-treated HepG2 cells showed a stepwise increase in subG<sub>1</sub> population, indicating that the reduction of MTT value in cisplatin-treated HepG2 cells was also due to growth arrest, but not to cell death alone. Western blot analysis revealed that p27, a member of the cyclin dependent kinase (CDK) inhibitor family, increased in hypoxic HepG2 cells while p21, another CDK inhibitor, decreased rapidly in hypoxic cells (Fig. 1(C)). On the other hand, the cisplatin-treated cells showed cell cycle arrest with an increase in p21 and p27, but the level of p16 was not changed. Therefore, hypoxia induced a delayed cell death (at 72 h), whereas cisplatin induced a gradual death of HepG2 cells.



**Fig. 1** Change in viability of HepG2 cells and analysis of cell cycle during hypoxia and cisplatin treatment. HepG2 cells were treated with hypoxia and cisplatin for 72 h. Hypoxic condition was near 0% (< 0.01%) of oxygen and cisplatin was treated at indicated concentration of 2 and 16 µg/ml. (A) Viability was measured by MTT analysis. (B) SubG<sub>1</sub> population and cell cycle analysis were performed by flow cytometry with propidium iodide staining. (a. control, b. 24 h hypoxia,

c. 48 h hypoxia, d. 72 h hypoxia, e. 24 h cisplatin at 2 µg/ml, f. 72 h cisplatin at 2 µg/ml, g. 24 h cisplatin at 16 µg/ml, h. 72 h cisplatin at 16 µg/ml). (C) Western blot analysis of cyclin-dependent kinase inhibitors at indicated time and doses. Actin represents a loading control. All experiments repeated at least three times. The data are expressed as mean ± SD



**Fig. 2** Analysis of cell death patterns through DNA fragmentation and western blot analysis of apoptotic molecules in hypoxia and cisplatin-treated cells. Whole cell lysate in (B) and (D) and cytosolic supernatant in (C) were analysed by Western blot. The arrowhead represents each indicated band. (A) DNA fragmentation pattern in hypoxia and cis-

platin. Far left lane designates 100 bp marker. (B) Western blot analysis of apoptotic proteins for mitochondrial and receptor-mediated apoptotic pathway. (C) Western blot analysis of cytochrome C in cytosol to test integrity of mitochondria. (D) Western blot of caspase-3 and PARP. (E) *in vitro* PARP cleavage assay

The profile of pro- or anti-apoptotic proteins in hypoxia and cisplatin-induced cell death

DNA fragmentation assay showed DNA laddering in both hypoxia and cisplatin-treated HepG2 cells (indicating apoptosis) in a dose and time dependent manner (Fig. 2(A)). However, hypoxia and cisplatin induced different patterns of apoptosis as shown in Fig. 1. Hence, western blot analysis was performed to test the levels of pro/anti-apoptotic molecules (Fig. 2(B)). The expression of p53, an effector protein of DNA damage, increased after low and high dose treatment of cisplatin. However, in hypoxic HepG2 cells, no change in p53 was seen in contrast to previous reports [14, 15]. The level of p53 was even decreased after 48 h of hypoxia. The level of Bax, which increases by p53 activation, was not changed in hypoxia. The level of p21, another target protein of p53 was not changed in hypoxia-treated HepG2 cells either (Fig. 1(C)). Bcl-X<sub>L</sub>, an anti-apoptotic protein on the mitochondrial outer membrane, decreased gradually as procaspase-9 decreased. However, in cisplatin-treated HepG2 cells, the level of Bax increased and the level of Bcl-X<sub>L</sub> was higher in low dose cisplatin-treated cells and

lower in high dose treated cells than in the untreated cells, as reported previously [23]. In addition, these activations of the mitochondria-mediated pathway were confirmed by the cytosolic release of cytochrome C (Fig. 2(C)). Procaspase-8, whose cleavage results in the activation of death receptor-mediated pathway, was cleaved or disappeared in hypoxia, but not in cisplatin-treated cells.

Next, we examined procaspase-3 and poly ADP-ribosyl polymerase (PARP) because caspase-3 is a final effector protein of both the mitochondria- and receptor-mediated pathways and because PARP is typically cleaved from 119 kD to 89 kD by activated caspase-3. In hypoxia, the cleavage of procaspase-3 was initially seen at 24 h after treatment and disappeared completely at 48 h. PARP was cleaved at 24 h, but full (119 kD) and cleaved (89 kD) bands disappeared gradually at 48 h after treatment. However, the cisplatin-treated HepG2 cells showed that the level of procaspase-3 decreased slightly by cisplatin treatment, but PARP was cleaved initially and continuously during cell death (Fig. 2(D)). Finally, to confirm the direct association between PARP cleavage and caspase-3 activity, we performed *in vitro* PARP cleavage assay. Only hypoxic cytosol at 24 h cleaved



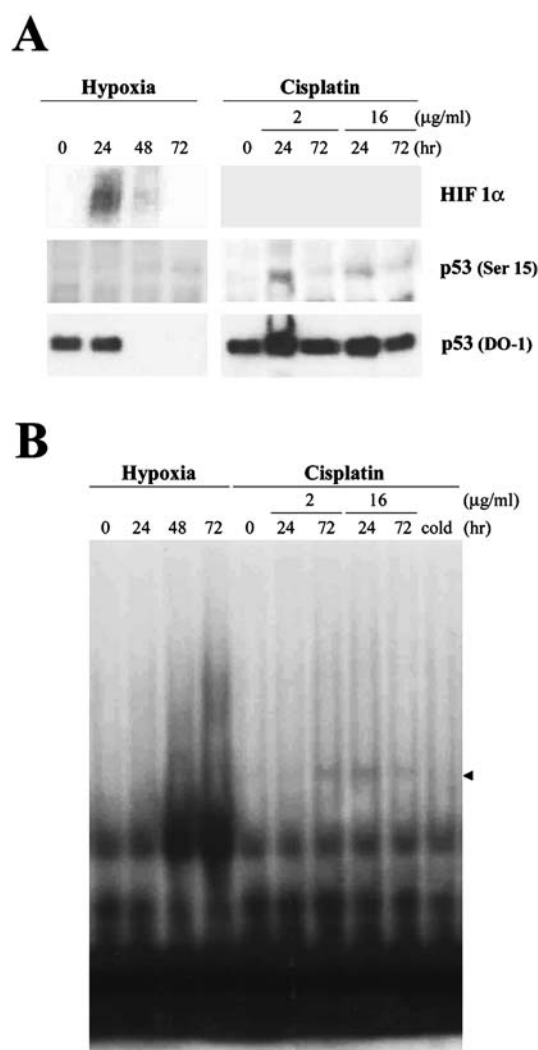
PARP in the nuclear extract while hypoxic cytosol at 72 h did not. Moreover, we found that hypoxic cytosol at 72 h degrade PARP without the cleavage to 89 kD. These results indicate that caspase apoptotic cascade in responses to hypoxia is different from that in response to cisplatin in that the hypoxic cells were initially resistant to apoptotic cell death, in spite of caspase-3 and PARP cleavages or breakdowns.

The difference of p53 status in hypoxic and cisplatin-treated HepG2 cells

Previously, it was reported that the hypoxic stimuli physiologically induce p53 [14, 15] and the early accumulation or phosphorylation of p53, despite some controversy [13]. However, our results show that the level of p53 was not increased and that the viability of HepG2 cells was even decreased as p53 disappeared. Therefore, we examined phosphorylation of p53 at serine 15, the most common phosphorylation site by genotoxic and non-genotoxic stress, and DNA binding activity of p53 (Fig. 3(A) and (B)). The cisplatin-treated HepG2 cells showed phosphorylation of p53 at serine 15 and accumulation of p53 (Fig. 3(A)). This result was supported by the observations of increases in p21 and Bax, the transcriptional target proteins of p53. In contrast to cisplatin, hypoxia induced no phosphorylation at serine 15 and did not cause accumulation of p53. HIF-1 $\alpha$ , a candidate stabilizer of p53 in hypoxia, was increased at 24 and 48 h after hypoxia treatment, suggesting no correlation between HIF-1 $\alpha$  and p53 in hypoxia. Furthermore, p53 in hypoxic HepG2 cells did not activate the downstream target proteins, p21 and bax. EMSA analysis showed that only the cisplatin-treated HepG2 cells had DNA binding affinity to the p53 binding site (Fig. 3(B)). According to these results, p53 in hypoxic cell death of HepG2 is not associated with the maintenance of apoptosis in contrast to cisplatin-induced cell death.

The effects of caspase inhibition on hypoxia and cisplatin treated HepG2 cells

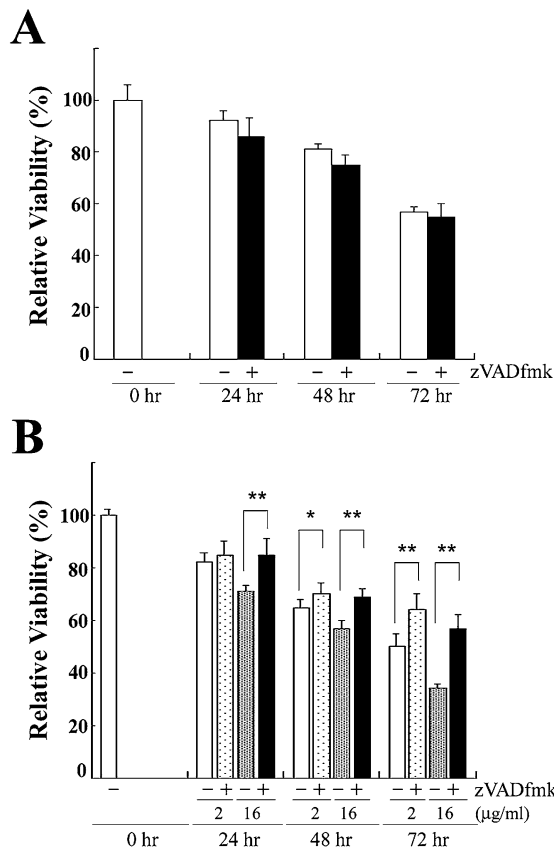
Both the hypoxia and cisplatin-treated HepG2 cells seemed to have reduced levels of some procaspases indicating activation of caspases (Fig. 2(B) and (C)); caspase-9 and caspase-3 after cisplatin treatment, and caspase-9, caspase-8, and caspase-3 in hypoxia. To confirm these observations, the treatment of the pan-caspase inhibitor, zVAD-fmk, under both conditions was tested (Fig. 4(A) and (B)). Interestingly, the responses were quite different. In cisplatin-treated cells, the viability measured through MTT reduction assay was reversed partially by zVAD-fmk. This partial reversal indicates that zVAD-fmk could not recover the cell cycle arrest. But, in hypoxia, viability was unchanged by zVAD-fmk. These results suggest that hypoxia-induced HepG2 cell death is not associated with caspase-dependent pathway.



**Fig. 3** The difference of p53 status in hypoxia and cisplatin-treated HepG2 cells. (A) The phosphorylation of p53 at serine 15 was examined by western blot analysis using serine 15 phospho-specific antibody initially and then with DO-1 p53 antibody to find p53 accumulation. HIF-1 $\alpha$  is shown as hypoxic indicator (B) Electromobility shift assay (EMSA) of p53. 'cold' at far right lane is a control that unlabeled probe was added to the 24 h with high dose of cisplatin sample. The arrowhead indicates DNA bound p53

#### Involvement of calpain in hypoxia-induced cell death

To investigate other differences between hypoxia and cisplatin treatments, we examined the levels of calpains, especially  $\mu$ -calpain and m-calpain, which are ubiquitously expressed (Fig. 5(A)). Initially, the cleavage of  $\mu$ -calpain from 84 kD to 65 kD was observed at 24 h after hypoxia treatment and the cleavage of m-calpain at 72 h in hypoxic HepG2 cells. However, no calpain cleavage was observed in cisplatin-treated cells. This result explains that the additional 18kD band of bax (from 21kD full band) on western blot is possibly a cleavage product by calpain in Fig. 2(B) [24]. Hypoxia induced possibly early cleavage of bax by calpain, but



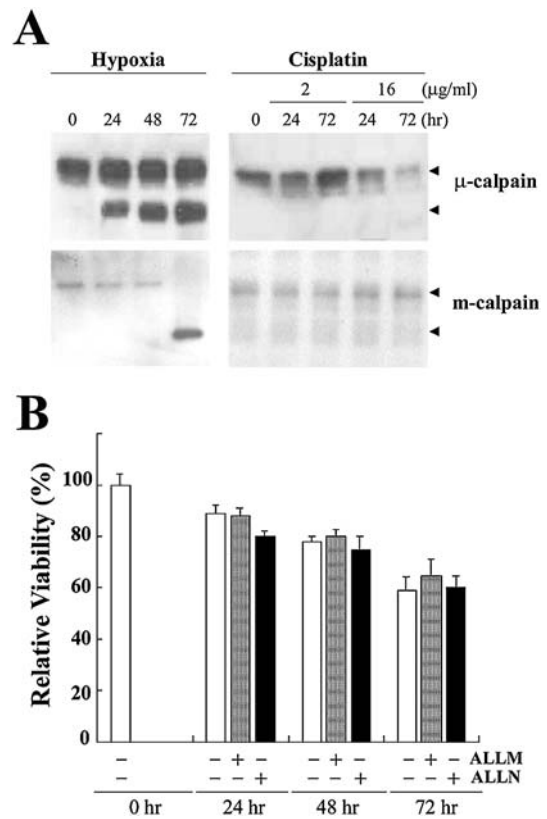
**Fig. 4** The effect of caspase inhibition by a caspase inhibitor on viability of hypoxia and cisplatin-treated HepG2 cells. HepG2 cells were treated with 100  $\mu$ M zVADfmk at 2 h before hypoxia or cisplatin treatment. The cell viability was measured by MTT assay as described in Materials and Methods. All experiments repeated at least three times. (A) Caspase inhibition by zVADfmk in hypoxia-treated HepG2 cells. (B) Caspase inhibition by zVADfmk in cisplatin-treated HepG2 cells at 2 and 16  $\mu$ g/ml. The data are expressed as mean  $\pm$  SD. Statistical significance between the group, \* $p < 0.05$ ; \*\* $p < 0.01$

cisplatin did not cause any bax cleavage even at a high dose at 72 h.

To test the effects of calpain inhibition, the calpain inhibitors, ALLM and ALLN, which can block both  $\mu$ -calpain and m-calpain, were treated (Fig. 5(B)). In hypoxia treated HepG2 cells, no change in viability measured by MTT analysis was observed. Therefore, caspase and calpain inhibitors had no effect on the viability of hypoxia-treated HepG2 cells.

The effect of calpain inhibitors on proteins involved in hypoxic cell death

The levels and cleavage patterns of the two calpains were analyzed by western blotting in hypoxic HepG2 cells treated with calpain inhibitors (Fig. 6(A)). The activities of  $\mu$ -calpain and m-calpain were decreased by two calpain inhibitors; ALLN inhibited cleavage of  $\mu$ -calpain more than ALLM did at 72 h after hypoxia treatment, whereas

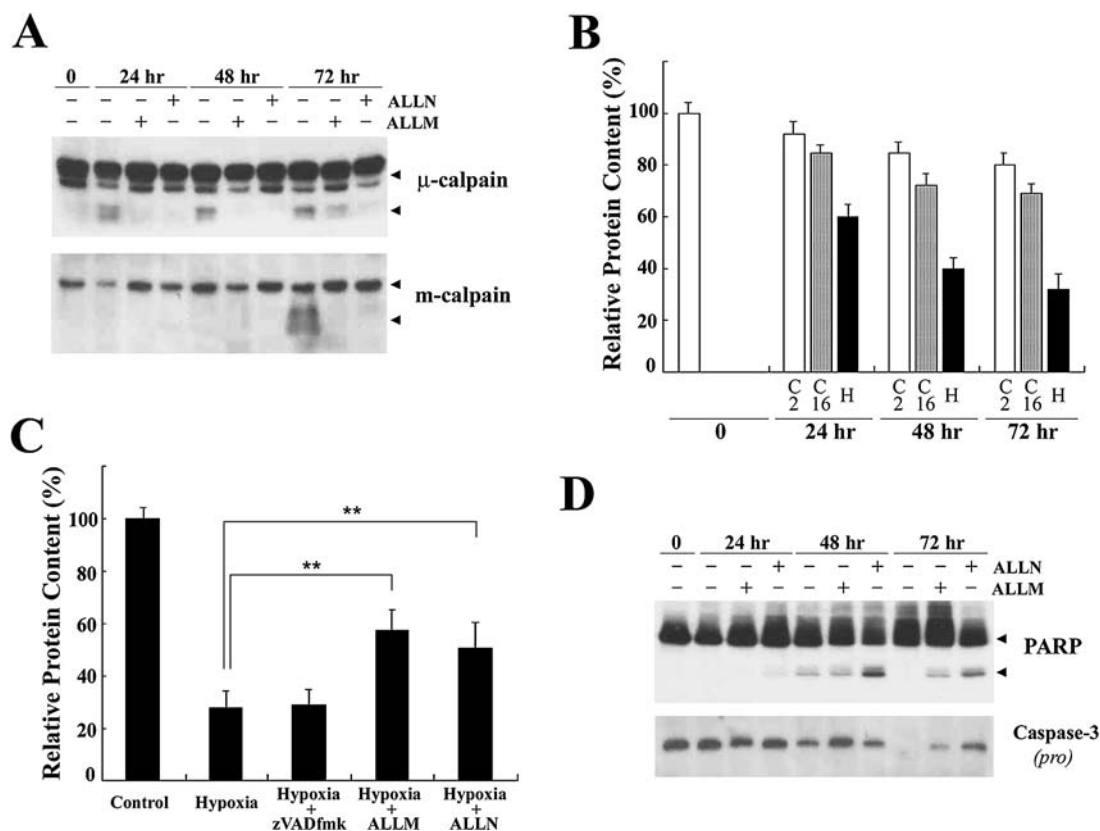


**Fig. 5** Calpain activities in hypoxia and cisplatin-treated HepG2 cells and the effect of calpain inhibition during hypoxia. Calpain inhibitors, ALLM and ALLN were added at 2 h before hypoxia treatment at a dose of 50  $\mu$ g/ml. (A) Activity of  $\mu$ -calpain and m-calpain in hypoxia and cisplatin-treated HepG2 cells analyzed by Western blotting. The two arrowheads represent full (or pro-) and cleaved bands. (B) The changes in viability by calpain inhibition in hypoxia-treated cells. The cell viability was measured by MTT assay

ALLM and ALLN inhibited m-calpain cleavage to the same extent.

Next, in order to measure proteolysis in hypoxia- and cisplatin-induced cell death, protein contents were examined using methylene blue staining, since proteolysis might be a major feature of cell death such as necrosis (Fig. 6(B)). The protein contents of cisplatin-treated cells were decreased slightly, but hypoxic cells showed a rapid loss in protein content. This result suggests that cell death of hypoxic HepG2 may be proteolytic in nature. We also examined the effect of calpain inhibitor and caspase inhibitor on proteolysis by hypoxia. Calpain inhibitors but not caspase inhibitor had a proteolysis-inhibiting effect (Fig. 6(C)).

Since calpain inhibition in hypoxic HepG2 cells may switch proteolytic cell death to apoptotic cell death, we attempted to determine whether calpain inhibition affects caspase activity. Western blot analysis revealed that the observed decrease in procaspase-3 at 72 h of hypoxia was reversed and that PARP cleavage, which represents the activation of caspase-3, was restored by ALLN and ALLM (Fig. 6(D)).



**Fig. 6** The effects of calpain inhibitors on proteolysis and cleavage patterns in hypoxia-induced HepG2 cell death. The cleavage of two calpains and PARP during hypoxia was examined by western blot after the treatment of ALLM and ALLN. In (B) and (C), protein content was determined by Methylene Blue methods. Simultaneously, MTT analysis was performed to compare with the Methylene Blue assay. Other procedures were described in materials and methods. Arrowheads indicate full and cleaved protein products. (A) Inhibition of  $\mu$ -calpain and m-calpain by ALLM and ALLN. (B) The effect of calpain inhibition on protein content as a result of proteolysis in hypoxia-induced HepG2 cell death (C: cisplatin at 2 and 16  $\mu$ g/ml, H: hypoxia). (C) Inhibition of proteolysis by calpain inhibitors. (D) PARP and caspase-3 after the treatment of calpain inhibitors in hypoxia-treated HepG2 cells. This experiment was performed after plating with low confluency (30–40%) of HepG2 cells to maximize the restoration of PARP cleavage by calpain inhibition. The data are expressed as mean  $\pm$  SD. Statistical significance between the group, \* $p < 0.05$ ; \*\* $p < 0.01$

The inhibition of calpain in hypoxia-treated cells prevented cleavages of caspase-3 and PARP. This result suggests that the abrogation of procaspase-3 by hypoxia may differ mechanistically from the reduction in procaspase-3 caused by the activation of caspase-3. Caspase-3 is a target of activated calpain, which attenuates both procaspase-3 and activated caspase-3 levels, as reported previously [25]. Thus, calpain in hypoxia seems to act by inactivating caspase-3 via proteolytic digestion.

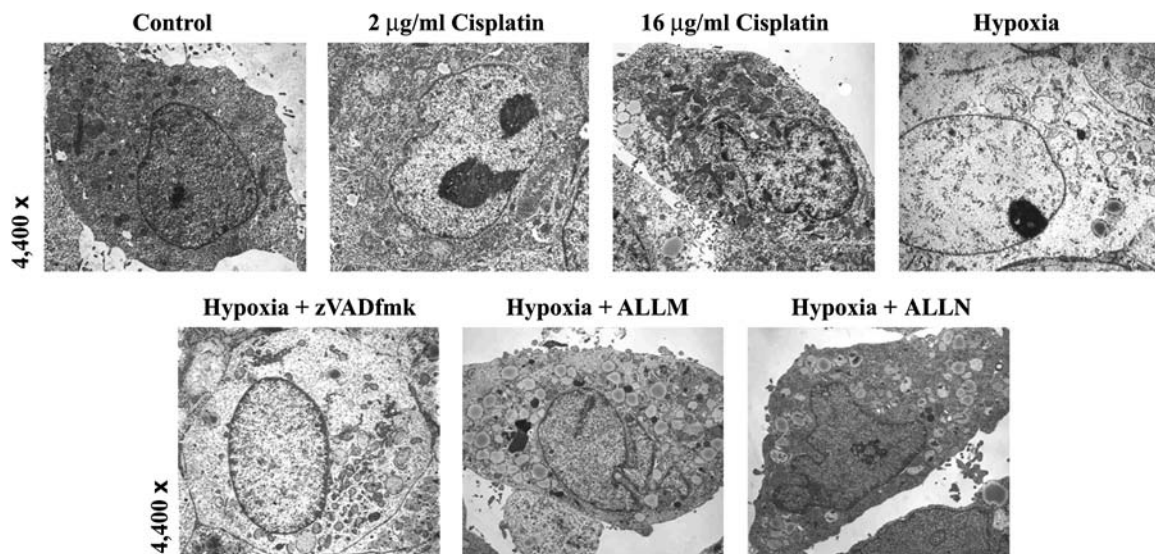
The effect of calpain inhibitors on the morphological changes induced by hypoxia

There has been some debate on the morphology of hypoxia-induced cell death, since the hypoxic cell death possesses both of apoptotic and necrotic features [26]. Therefore, we analyzed the morphologies of hypoxic cells by transmission electron microscopy (Fig. 7). The morphology of cisplatin-treated HepG2 cells, as a control of apoptotic cells, showed

nuclear segmentation or condensation and swelling of intracellular organelle with intact membrane integrity, even at high dose (16  $\mu$ g/ml). However, the hypoxic HepG2 cells showed some differences; cellular membrane swelling, scanty cytoplasm, severe swelling of mitochondria, and marginal nuclear condensation. In addition, the structure of endoplasmic reticulum in hypoxic HepG2 cells was completely destroyed, in contrast to other organelles, which were preserved both in shape and number. This morphology in hypoxia is more likely to be necrotic than apoptotic, although the presence of DNA fragmentation in Fig. 2(A) suggests some apoptotic feature of cell death.

To determine whether inhibitors of caspase or calpain could change these morphological patterns, we added these inhibitors into hypoxia- and cisplatin-treated cells. Treatment with zVADfmk, a caspase inhibitor, did not alter cell morphology in hypoxic cells. However, the HepG2 cells treated with the calpain inhibitors, ALLM and ALLN, showed some morphological changes compared to the untreated cells. The





**Fig. 7** The morphological change of hypoxia and cisplatin-treated HepG2 cells after treatment of calpain or caspase inhibitor(s). Hypoxia was treated for 72 h and cisplatin was treated at 2 and 16  $\mu\text{g/ml}$  for 72 h. A caspase inhibitor, zVADfmk (100  $\mu\text{M}$ ) and calpain inhibitors, ALLM

and ALLN (50  $\mu\text{g/ml}$ ), were added at 2 h before hypoxia or cisplatin treatment. The morphology of the HepG2 cells was examined by transmission electron microscopy as described in Materials and Methods. These photographs are represented at 4,400 fold magnifications

morphology appeared to be less necrotic after calpain inhibition than in the hypoxic state (e.g. ER structure is preserved) though cytosolic vacuolation indicating necrosis was still existed. Nuclei in hypoxic cells treated with calpain inhibitors was shrunken or did not show marginal fragmentation, which was absent in the hypoxic state alone, though this was not similar to the nuclear morphology seen in cisplatin-treated cells as the control of apoptotic morphology. This nuclear morphology appears to be due to the partial activation of caspase cascade after calpain inhibition.

## Discussion

### The role of p53 in hypoxia-induced cell death

To examine the mechanism of cell death induced by hypoxia, we compared hypoxic cells and cisplatin-treated HepG2 cells. Compared to the role of p53 in genotoxic stress such as cisplatin treatment, hypoxia is a unique stress in that it modulates p53 protein by stabilization and phosphorylation [27]. However, it has been reported that the activation of p53 does not appear to occur only through phosphorylation at the serine 15 position and this phosphorylation and p53 accumulation appear to be unrelated [28]. It was also argued that hypoxia-induced p53 is transcriptionally impaired [29]. Therefore, we investigated the relationship between hypoxia-induced cell death and function of p53. Our results show that hypoxia did not induce either accumulation of p53 or phosphorylation of p53 at serine 15 in HepG2 cells. On the other hand, cisplatin induced phosphorylation and accumulation

of p53. Increase in p53 downstream target proteins, such as p21 and Bax, were also apparent, as expected, in the cisplatin treated cells. Thus, we concluded that the role of p53 in the hypoxia-induced death of HepG2 is not as important as in cisplatin-induced cell death.

### The role of caspase and calpain in hypoxia-induced cell death

Caspases are important mediators of apoptotic processes and caspase-3 is a common downstream effector. Caspase-3 exists as a proenzyme in most cells and is activated by caspase-9 or caspase-8 via two distinct pathways. First, in mitochondrial pathway, a complex of mitochondrially released cytochrome C and apoptotic protease-activating factor 1 (APAF1) with caspase-9 leads to caspase-3 dimerization and autolytic activation, which in turn processes and activates procaspase-3. Second, in receptor-mediated pathway, activated death-domain containing receptors, such as Fas, recruit adapter proteins, which also contain FADD (Fas-associated death-domain). Receptor-associated FADD then induces the autolytic activation of receptor-associated caspase-8, which activates caspase-3 proteolytically. In this study, hypoxia induced the reduction of procaspase-8, -9, and -3, indicating both pathways were involved. But, apoptosis of cisplatin-treated HepG2 was activated only by mitochondrial pathway via caspase-9.

Another apoptotic target, calpain is a ubiquitous cysteine protease with two major isoforms,  $\mu$ - and m-calpain, which have recently been shown to be activated by a number of apoptosis-inducing agents, such as ionizing

irradiation, etoposide, or staurosporine [20, 30, 31]. The potential role of calpains in apoptosis is also indicated by growing list of calpain substrates; p53, I $\kappa$ B $\alpha$ , PARP, and several cytoskeletal proteins including fodrin and  $\alpha$ II-spectrin [32, 33]. Moreover, calpains were found to be involved in the cleavage of caspase-3, -7, -8, and -9 [34, 35]. Though numerous results indicate that calpains are involved in apoptosis, exact relationship between calpain and caspase activation has not been demonstrated clearly. One possible relationship is inactivation of caspases by calpains [36, 37]. Our study showed that the levels of many proteins such as p53, Bcl-X<sub>L</sub>, procaspase-8, procaspase-9, and procaspase-3 were reduced in hypoxic HepG2 cells, but not in cisplatin treated HepG2 cells. Furthermore, cleavage of PARP, indicating the activation of caspase-3, occurred at early stage when cell death had not been induced and procaspase-3 attenuation was not prominent. In other words, the attenuation of procaspases in hypoxia-induced HepG2 cell death indicates the inhibition of caspase activation. Thus, we speculated that the attenuation of caspase-8 and caspase-9 in hypoxic cells was not resulted from the activation of both the mitochondrial and receptor mediated pathways, but from the inactivation of caspases by calpains. This was supported by proteolytic nature of hypoxia treated cells that were not shown in cisplatin-treated cells. Cellular protein content was dramatically reduced in hypoxia treated cells. This seems to be mediated by the activation of calpains, because calpain inhibition, but not caspase inhibition, rescued this proteolysis (Fig. 6(C)) [38]. In addition, competition between caspases and calpains might occur in hypoxia treated cells. Caspases were activated at the beginning of hypoxia but soon calpains dominated and cleaved various cellular proteins to reduce the content of cellular protein. In particular, this point was also identified in cytochrome C release of hypoxic HepG2, which was shown in Fig. 2(C) with the caspase-3 activity in Fig. 2(D) and (E). As mentioned above, cytochrome C is released before caspase-3 activation in classical apoptosis. However, in necrosis, the release of cytochrome C is caspase-independent and the extent of the release is more profound because of mitochondrial rupture instead of just mitochondrial swelling or minor rupture in apoptosis [39, 40]. Thus, we concluded that the massive release of cytochrome C of hypoxic HepG2 at 72 h was due to necrosis, while the slight release at 24 h showed apoptosis accompanied by caspase-3 activation. Finally, calpain inhibition shifted to caspase-dependent pathway without changing viability, indicating that it blocked only one of these pathways and it was ineffective at rescuing cells from hypoxic insult.

Indeed, calpain was reported as a major molecule that induces necrotic cell death by hypoxia. Aki et al. showed calpain-mediated necrosis in hypoxia-treated cardiomyocytes, biochemically and morphologically [41, 42]. In their conditions, necrosis was inhibited by the calpain inhibitor

SJA6017 peptide in a calcium-dependent manner that endogenous calcium chelator BAPTA-AM blocked cell death, which was checked by erythrosin B exclusion assay, and fodrin cleavage. In our conditions, calpain-induced fodrin cleavage was also inhibited by BAPTA-AM and EGTA, endogenous and exogenous calcium chelators, respectively (*data not shown*). However, in comparison to the reports of Aki et al. the treatment of calpain inhibitors, ALLM or ALLN, into hypoxic HepG2 cells did not show the change of viability by MTT analysis despite the inhibition of proteolysis. This discrepancy can be explained as follows; (i) Cellular responses between cardiomyocyte and hepatocyte might be different. (ii) The methods to measure the viability, MTT and erythrosin B analysis, are different, in that MTT analysis measures a mitochondrial respiration and erythrosin B exclusion assay indicates the membrane integrity of live cells. (iii) The use of more potent or specific calpain inhibitors may improve the viability tests in our experiment. In fact, although ALLM and ALLN are generally used as inhibitors of calpains, they have other effects since ALLN has some characteristics of proteasome inhibitors. There has been some reports about several calpain inhibitors with the higher potency for calpain such as MDL28170 and further treatment of these to measure the effect of hypoxia insult would be valuable [43].

#### The morphology of hypoxia-induced cell death

Shrinkage and fragmentation of the nucleus and cell body, and extensive degradation of chromosomal DNA are the most characteristic features of apoptosis. Our results show that the morphology of cisplatin-treated cells is a typical of apoptosis, suggesting caspase-dependent death. In contrast to cisplatin-treated cells, the hypoxic HepG2 cells exhibited near necrotic cell death. Recent reports have suggested that the blockage of caspase cascade by Apaf-1 gene knock out [44, 45] and genetic deletion of caspase-3 and/or caspase-9 [46] can change the cell death pattern from classical apoptosis to an 'alternative' death mode; so called, 'apoptotic necrosis', 'non-apoptotic programmed cell death', 'paraptosis' or 'aponecrosis' [47]. Calpain inhibition by ALLN and ALLM apparently restored the activity of caspase-3, which cleaves PARP from 119 kD to 89 kD. These two calpain inhibitors also inhibited the proteolysis of hypoxia-treated HepG2 cells. Morphologically, the inhibition of calpain resulted in partial nuclear segmentation and shrinkage in hypoxic HepG2 cells, which were not seen in hypoxic state.

In addition, the morphology due to hypoxia alone showed greater damage to ER than other organelles, e.g., mitochondria, a general target of classical apoptosis was preserved. Recently, several reports have suggested that ER stress signals may induce apoptosis, and calpain was activated by ER stress [48–50]. But, the upstream signals of calpain

activation have not been identified and the initiation of ER stress has not been investigated. Moreover, little is known how calpain in hypoxia treated cells is associated with ER stress [50].

#### DNA fragmentation in hypoxia-induced cell death

Recently, an endonuclease was reported to be activated specifically by caspase-3 during apoptosis in human and mice. The human gene for this protein has been named caspase-activated nuclease or DFF40 (DNA fragmentation factor, 40 kDa subunit) [51], whereas the mouse homologue is called caspase-activated DNase (CAD) 1 [52]. CAD is maintained as an inactive form in the cytoplasm of normal cells by association with a protein that acts as a chaperone and an inhibitor. This protein is called inhibitor of CAD (ICAD) in mice or DFF45 (DNA fragmentation factor, 45 kDa subunit) in humans [53]. However, recent reports suggest that the DNA fragmentation and chromosomal condensation involve several molecules in addition to CAD, such as Acinus (apoptosis chromatin condensation inducer in the nucleus), AIF (apoptosis inducing factor), L-Dnase II, and cathepsin B [54]. These factors are usually associated with the activation of caspases. For example, partial or complete chromatin condensation is observed when the tumor suppressor gene PML is overexpressed in the absence of caspase activation [55]. On the other hand, cathepsin B induces peripheral condensation of nucleus, whereas other factors cause homogeneous karyorrhexis or pyknosis [56]. We believe that the morphologically incomplete reversal of hypoxic cells into apoptotic morphology by calpain inhibition is related to the unusual fragmentation induced by other factors such as cathepsin B despite the dramatic shift to caspase-dependent apoptosis evidenced by PARP cleavage patterns. However, the detailed mechanism involved should be elucidated by further investigation.

#### Conclusion

It is apparent that the calpain signal is more important in hypoxia-induced cell death than in DNA damage induced by cisplatin. Morphologically, hypoxia confers a typical necrosis-like cell death and calpain inhibition recovered caspase activity and reversed morphological changes into apoptosis to some extent without affecting viability. Our results suggest that the maintenance of hypoxic cell death is associated more tightly with calpain activity than caspase through proteolysis of many effector proteins of apoptosis. It is now believed that apoptosis occurs at the beginning of hypoxic cell death, but soon calpain dominates this process, which is biochemically and morphologically close to necrosis in spite of the presence of DNA fragmentation. The present study

identified the stepwise processes of cell death, and some intrinsic characteristics of hypoxia-treated cells that would contribute to further understanding of molecular signaling induced by hypoxia. This information may provide molecular basis for the treatment of hypoxic tumors or brain or cardiovascular ischemia.

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