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Effect of Hypoxia on the Signal Transduction of Apoptosis in Osteoblasts

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Mammalian cell is critically dependent on a continuous supply of oxygen. Even brief periods of oxygen deprivation can result in profound cellular damage. The aim of this study was to examine the possible mechanism of apoptosis in response to hypoxia in MC3T3E1 osteoblasts.

MC3T3E1 osteoblasts under hypoxic conditions (2% oxygen) resulted in apoptosis in a time-dependent manner, determined by DNA fragmentation assay and nuclear morphology, stained with fluorescent dye (Hoechst 33258) Pretreatment with Z-VAD-FMK, a pan-caspase inhibitor, or Z-DEVD-CHO, a specific caspase-3 inhibitor, suppressed the DNA ladder in response to hypoxia in a concentration-dependent manner. An increase in caspase-3-like protease (DEVDase) activity was observed during apoptosis, but no caspase-1 activity (YVADase) was detected. To confirm what caspases were involved in apoptosis, western blot analysis was performed using an anti-caspase-3 or 6 antibody. The 17-kDa protein, that corresponds to the active products of caspase-3 and the 20-kDa protein of the active protein of caspase-6 were generated in hypoxia-challenged lysates, in which the full length forms of caspase-3 and 6 were evident. With a time course similar to caspase-3 and 6 activation, hypoxic stress also caused the cleavage of Lamin A, typical of caspase-6 activity. In addition, the hypoxic stress elicited the release of cytochrome c into the cytosol during apoptosis. These findings suggested that the activation of caspases accompanied by a cytochrome c release in response to hypoxia was involved in apoptotic cell death in MC3T3E1 osteoblasts.

Key words : Hypoxia, Apoptosis, Osteoblast

There are two distinct essential morphological manifestations of cell death; one is apoptosis and the other is necrosis. Apoptosis is characterized by the condensation of chromatin and cytoplasm, membrane blebbing and apoptotic bodies, whereas necrosis features plasma membrane disruption and mitochondrial swelling associated with formation of large vacuoles.[1-3] However, the specific intracellular pathways by which this apoptosis occurs are not well delineated.

Hypoxia itself causes apoptotic cell death in human fibroblast cell line GM701, human lymphoid cell line SKW6.4, murine pro-B cell line BAF 3, rat hepatoma cell line 7316A, and rat pheochromocytoma cell line PC12 in vitro.[4,5]

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Caspases, a family of cysteine proteases, play a critical role in the execution phase of apoptosis and are responsible for many of the biochemical and morphological changes associated with apoptosis.[6] Caspases are the principal effectors of apoptosis.[7] These cysteine proteases reside in the cytosol of all animal cells as inactive zymogens. Proteolytic processing of these zymogens generates active enzymes and triggers apoptosis. A variety of experimental approaches, including use of cell-permeable peptidyl inhibitors and genetically engineered mice, have demonstrated an important role for caspases in neuronal cell death in vivo after ischemic insults.[8,9] It has been proposed that initiator caspases with long prodomains, such as caspase-8 (MACH/FLICE/Mch 5), either directly or indirectly activate effector caspases, such as caspase-3, 6, and 7.[10] These effector caspases then cleave intracellular substrates, such as poly (ADP-ribose) polymerase (PARP) and lamins, during execution phase of apoptosis.[11] As caspase-8 can activate all known caspases in vitro, it is a prime candidate for an initiator caspase in many forms of apoptosis.[12] Pro-caspase-9 has also been proposed as an initiator caspase; in the presence of dATP and cytochrome c, its long N-terminal domain interacts with Apaf-1 resulting in the activation of caspase-9.[13,14] Active caspase-9 can then activate the effector, caspase-3, 6, and 7.[15] Thus there are at least two major mechanisms by which a caspase cascade resulting in the activation of effector caspases may be initiated as follows: one involving caspase-8 and the other involving caspase-9 as the most apical caspase.

Cytochrome c, which is usually present in the mitochondrial inter-membrane space, is released into the cytosol following the induction of apoptosis by many different stimuli.[16] Release of mitochondrial cytochrome c and activation of caspase-3 is blocked by the anti-apoptotic members of the Bcl 2 family, such as Bcl 2 and Bcl-XL, and is promoted by pro-apoptotic members, such as Bax and Bak.[17,18]

It has been proposed that the release of cytochrome c and subsequent caspase activation are required in apoptosis. However, the signal transduction of apoptosis in osteoblasts is not well understood.

The purpose of this study was to examine whether hypoxia could induce apoptosis in MC3T3E1 osteoblasts, and to determine which intracellular signals triggered by hypoxic stress which resulted in cell death.

MATERIALS AND METHODS

1. Materials

Caspase-3, caspase-6, Lamin A and cytochrome c antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). GST-c-jun N-terminal proteins were purchased from Stratagene (La Jolla, CA, USA). Hoechst 33258 and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphnyltetrazolium bromide (MTT) were purchased from Sigma Chemical Company (St. Louis, MO, USA). N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin(Ac-DEVD-AMC), N-acetyl-Tyr-Val-Ala-Asp-4-methyl-coumaryl-7-amide(Ac-YVAD-AMC), and pansorbin were obtained from Calbiochem-Behring Corp. (La Jolla, CA, USA). Z-Val-Ala-Asp-fluoromethylketone (ZVAD-FMK) was purchased from Kamiya Bio Company (Seattle, WA, USA). A genomic DNA purification kit was obtained from Promega (Madison, WI, USA). Lactate dehydrogenase kit and SDS-PAGE reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA). All cell culture media and reagents were obtained from Life Technologies, Inc.

2. Cell culture and hypoxic treatment

The clonal murine osteoblast cell line, MC3T3E1, was purchased from Riken Bank and cultured in α-modified essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. Our system for exposing cell cultures to hypoxia created defined atmospheric oxygen partial pressure (pO2) values within the approximate range of 2%. We defined the low oxygen conditions used for these studies as

hypoxia, rather than anoxia. The oxygen tension in the culture medium at 1 hour, 6 hours, and 48 hours after the transfer to the hypoxic chamber fell to 2.2, 2.0, and 0.2% respectively. When MC3T3E1 osteoblasts achieved 80% confluence, the hypoxic experiments were all performed at 2% of PO_2 in a mixture of 5% CO_2 and the balance N_2 in a humidified 37℃ incubator (ANX -1, Hirasawa, Tokyo, Japan). Atmospheric oxygen levels in this apparatus were calibrated using a polarographic oxygen electrode (Oxygen Sensors, Inc., Norriston, PA, USA).

3. MTT assay

The MTT assay of cell viability was performed as described previously.[19] Briefly, after exposure to hypoxia, MTT was added to cells for a final concentration of 0.5 mg/ml and incubated at 37℃ for 5 hours. The medium was aspirated, and the formazan product was dissolved with dimethylsulfoxide. Absorbance at 630 nm (backward absorbance) was subtracted from absorbance at 570 nm for each well.

4. LDH release

Cell viability was also assessed by measuring the release of intracellular lactate dehydrogenase (LDH). The sensitivity of LDH release in this model is comparable to a MTT assay. After treatment with control or hypoxic conditions, the maintenance medium was collected from each monolayer. MC3T3E1 cells were then washed with Hanks balanced salt solution, and collected by scrapping with a rubber policeman into a 10 mM Tris (hydroxymethyl) aminomethane HCl buffer (pH 7.4) containing 0.2% Triton X-100. Duplicate aliquots (40 μl) of the culture medium and cell lysates were placed in 96-well microtiter plates, and LDH activity was measured by monitoring the consumption of nicotinamide adenine dinucleotide at 340 nm using a kinetic spectrophotometric plate reader. Results are expressed as the percentage of the intracellular LDH released to the culture medium: % LDH release = [LDH activity in media/(LDH activity in cells + media)] x 100.

5. Agarose gel electrophorosis for DNA fragmentation

The characteristic ladder pattern of DNA breaks was analyzed by agarose gel electrophoresis. Briefly, DNA from the MC3T3E1 cells ($1x10^6$ cells/each group) was isolated by Wizard Genomic DNA purification kit (Promega Co., Wisconsin Medicine, WI, USA) and isolated by serial ethanol precipitation. Isolated genomic DNA (10 μg) was subjected to 1.5% of agarose electrophoresis at 100 V for 1 hour. DNA was made visible by staining with ethidium bromide under UV light.

6. Morphological Detection of Apoptosis

Morphological evaluation of apoptotic cell death was performed as described with some modification.[20] Cover slips were fixed for 5 min in 3% paraformaldehyde in phosphate-buffered saline. After air-drying, the cover slips were stained for 10 minutes in Hoechst 33258 (10 μg/ml), mounted in 50% glycerol containing 20 mM citric acid and 50 mM orthophosphate, and stored at -20℃ before analysis. Nuclear morphology was evaluated using a fluorescent microscope (Zeiss IM 35) at an excitation and emission wavelength of 440 and 460 nm respectively. Apoptotic cells were identified as those whose nucleus exhibited brightly stained and condensed chromatin or nuclear fragmentation.

7. Fluorogenic substrate assay for caspase activity

Cytosolic cell extracts were prepared by lysing the cells in a buffer containing 1% Nonidet P-40, 200 mM NaCl, 20 mM Tris/HCl (pH 7.4), 10 μg/ml leupeptin, aprotinin (0.27 trypsin inhibitor/U/ml) and 100 μM phenylmethylsulfonyl fluoride (PMSF). Caspase-1 or caspase-3-like activity was determined by incubation of the cell lysate (containing 25 μg of total protein) with 50 μM of the fluorogenic substrate, AC-YVAD-AMC or AC-

Table 1. MTT reduction and LDH relaease in MC3T3E1 osteoblasts in hypoxia (Mean±S.E.)

Time (hr)	MTT	LDH
0	100 ± 5.4	10.1 ± 4.8
24	96.2 ± 5.8	23.3 ± 3.9
30	92.1 ± 6.3	35.2 ± 4.3*
36	78.1 ± 4.9*	45.1 ± 5.1*
42	63.5 ± 5.1*	55.7 ± 5.4*
48	53.8 ± 5.9*	67.3 ± 4.6*
54	34.1 ± 5.5*	95.2 ± 4.9*

* : significantly different with the just previous data ($p < 0.05$)

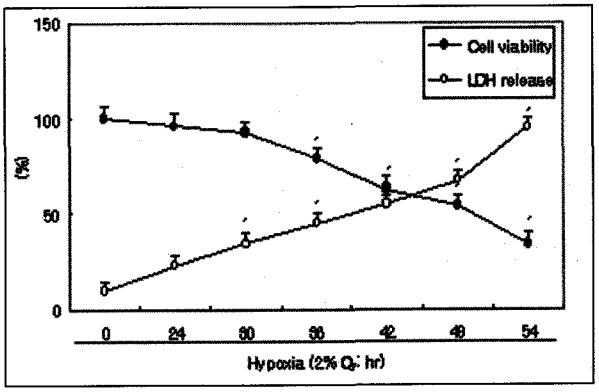

Fig. 1. Hypoxia induced cell death in MC3T3E1 osteoblasts. MC3T3E1 osteoblasts were subjected to hypoxia (0, 24, 30, 36, 42, 48, or 54 hour), followed by measurement of MTT reduction and LDH release as described in Materials and Methods. Data are expressed as the percent of controls that were not exposed to hypoxia. Mean ± S. E. (n=4). * Significantly different from control. P<0.05.

DEVD-AMC respectively, in a 200 μl cell-free system buffer, composed of 10 mM Hepes (pH 7.4), 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM pyruvate, 0.1 mM PMSF, and 1 mM dithiothreitol. The release of fluorescent 7-amino-4-methylcoumarin was measured for 1 hour at 2-minute intervals by spectrofluorometry.

8. Cytochrome c Measurements

Mitochondrial fractions were prepared from $1x10^7$ MC3T3E1 cells by differential centrifugation in a buffer containing 250 mM sucrose.[1] Protein samples of 25 μg were loaded in sodium dodecyl sulfate-15% polyacrylamide gels, subjected to electrophoresis, and then electrophoretically transferred to nitrocellulose membranes. Western blots were probed with primary monoclonal anti-cytochrome c antibodies (Pharmingen, San Diego, CAlif, USA) and secondary anti-mouse horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology, USA) and then developed with enhanced chemiluminescence (Amersham Life Science).

9. Data presentation and statistical analysis

Data were presented as mean±S.E. Students' t-test was used for statistical analysis. Results were considered to be significant at the 5% confidence level.

RESULTS

1. Hypoxia induced apoptotic cell death in MC3T3E1 cells

To address the ability of hypoxia to induce cell death, we investigated the effect of hypoxia on cell viability using MTT and LDH release assay kit. MC3T3E1 osteoblasts were found to be relatively resistant to hypoxia-induced cell death. A small loss of viability was observed within 36 hours of a hypoxia challenge. After 48 hours, loss of viability occurred following hypoxia in a time-dependent manner (Table 1, Fig. 1). At 48 hours, we employed two methods to investigate whether the loss of viability caused by hypoxia correlates with a biochemical feature that discriminates between apoptosis and necrosis. In order to characterize apoptosis, internucleosomal DNA fragmentation and nuclear morphology using Giemsa solution and Hoechst dye 33258 were examined (Fig. 2). Approximately, 35% of cells were apoptotic when evaluated by Hoechst 33258

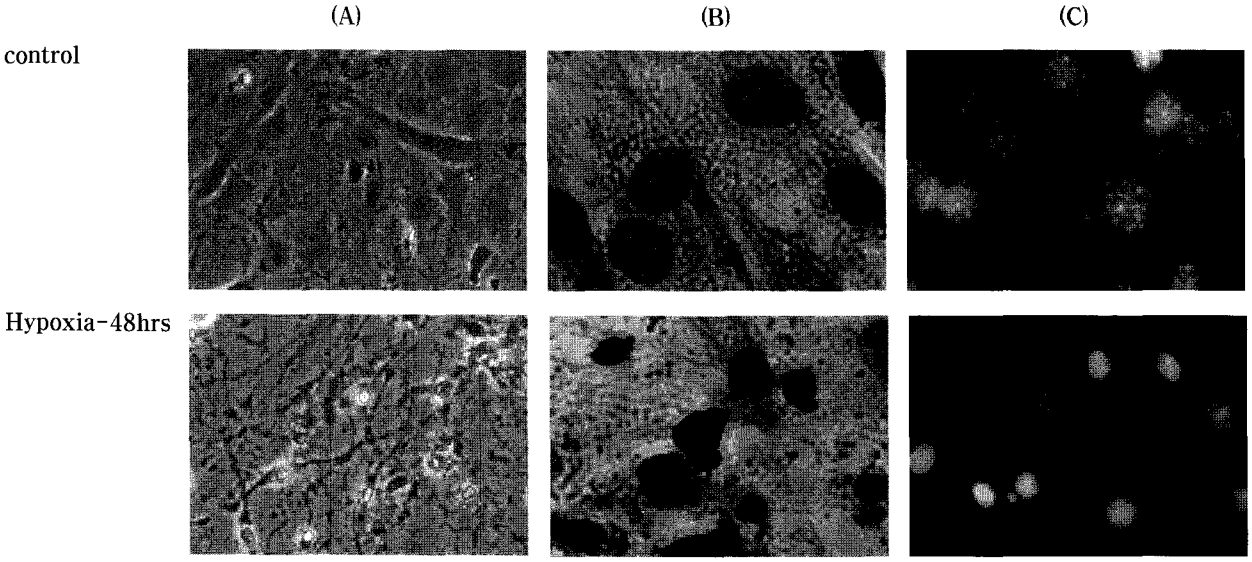

Fig. 2. Hypoxia induced apoptosis in MC3T3E1 osteoblasts.
(A). Morphological analysis by phase-contrast microscopy. Upper, untreated control cells; lower, hypoxia (48 hour)-treated cells (x100). (B). Programmed cell death in fixed cultured cells was assessed by nuclear morphology after staining with Giemsa solution. (C). Nuclei were assessed by Hoechsat 33258 staining in an Olympus OMT2 inverted fluorescence microscope, using a long working-distance objective, at a final magnification of x200. Left, untreated control cells; right, hypoxia (48 hours)-treated cells.

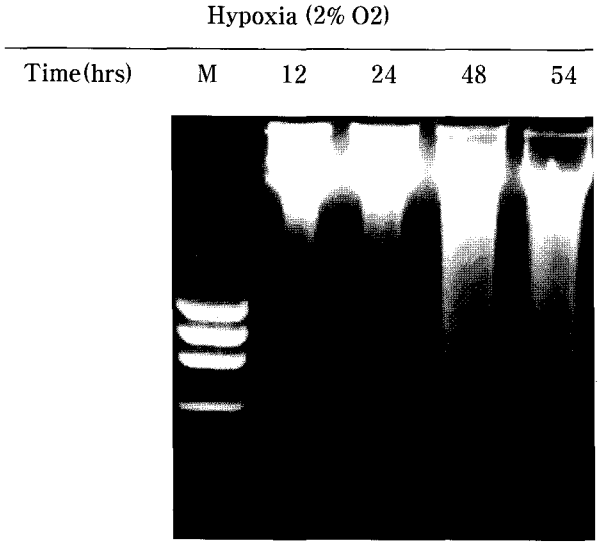

Fig. 3. Hypoxia induced DNA fragmentation in MC3T3E1 osteoblasts.
MC3T3E1 cells were subjected to hypoxia (0, 24, 48, or 54 hours), followed by the genomic DNA was isolated and separated on 1.5% agarose gels. The DNA was stained with ethidium bromide and visualized under UV light.

staining. Apoptotic cells were identified by their condensed and fragmented nuclei. A major component of hypoxia-induced death was attributable to apoptosis, as shown by DNA laddering. Significant DNA laddering was present after 48 hours of exposure in MC3T3E1 cells but was not present in the control group at the same time point (Fig. 3).

2. Hypoxia induced apoptotic cell death via caspase activation

The caspase family of proteases is thought to be the final execution pathway in apoptosis.[19,21] Having established a role for caspases in cell death induced by hypoxia, we analyzed the process of caspase activation within this system. As shown in Table 2 and Fig. 4, the fluorescence intensity of the caspase-3 cleavage product AMC was monitored to obtain a linear standard curve. However, The cystein-protease, caspase-1 was not activated for the hypoxia-induced apoptosis signal transduction pathway in MC3T3E1 cells. To establish the role of the caspase pathway in hypoxia-induced

Table 2. Caspase 3-like protease activation in MC3T3E1 osteoblasts in hypoxia (Mean±S.E.)

Time (hr)	Caspase-3	Caspase-1
0	0.05 ± 0.030	0.05 ± 0.027
24	0.09 ± 0.022	0.05 ± 0.021
30	0.15 ± 0.024*	0.06 ± 0.018
36	0.42 ± 0.025*	0.06 ± 0.022
42	0.59 ± 0.028*	0.06 ± 0.019
48	0.57 ± 0.031*	0.06 ± 0.024
54	0.51 ± 0.026*	0.06 ± 0.023

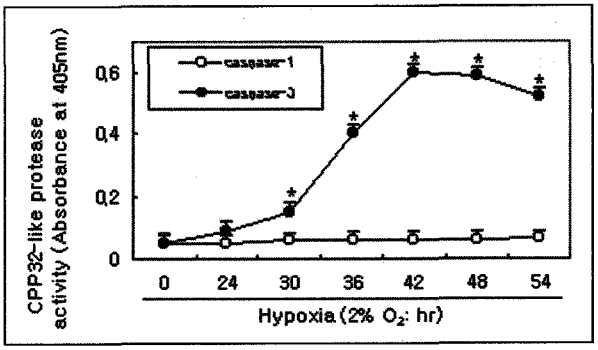

Fig. 4. Hypoxia induced caspase 3-like protease activation in MC3T3E1 osteoblasts.
MC3T3E1 cells were subjected to hypoxia (0, 24, 30, 36, 42, 48, or 54 hours), followed by caspase activity analysis as follows. The activity of caspase-1-and 3-like proteases was measured by proteolytic cleavage of substrates including 100mM of acetyl-YVAD-AMC and acetyl-DEVD-AMC, respectively. These fluorogenic substrates and AMC as a control were solubilized in an assay buffer containing 100 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 0.1 mM PMSF, 10 g/ml aprotinin, 1 g/ml leupeptin, and 2 mM dithiothreitol. The amounts of released 7-amino-4-methylcoumarin (AMC) were measured using spectrofluorometer with excitation at 380 nm and emission at 460 nm.

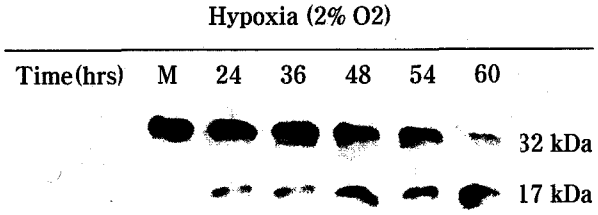

Fig. 5. Hypoxia induced caspase-3 proteolytic cleavage in MC3T3E1 osteoblasts.
MC3T3E1 cells were incubated for various periods in hypoxic environment. Total cell lysates were prepared and then subjected to western blot analysis as described in Materials and Methods using antibody for caspase-3.

apoptosis of osteoblasts, we treated MC3T3E1 cells with pan-caspase inhibitor Z-VAD-FMK (specific caspase-1 inhibitor), YVAD-CHO, or DEVD-CHO (specific caspase-3 inhibitor). The pretreatment of MC3T3E1 cells with Z-VAD-FMK or DEVD-CHO largely pre-vented cell death after 48 hours of hypoxia. The formation of a DNA ladder in cells 48 hours after the hypoxia challenge was suppressed by simultaneous treatment with 100 μM Z-DEVD-CHO or Z-VAD-FMK (data not shown). In addition, caspase-6 (but not caspase-3) has been shown to cleave the nuclear lamins that are critical to maintaining the integrity of the nuclear envelope and cellular morphology.

Caspase-3 and 6 are synthesized as precursor molecules, and are approximately 32-kDa and 34-kDa in size respectively.[22] During processing, caspase-3, 6 are proteolytically cleaved to produce a mature enzyme composed of each 17 and 20-kDa subunit respectively. First, to investigate whether or not hypoxia activates caspase-3, new bands corresponding to p17 of caspase-3 were detected at 24 hour after the hypoxic challenge in samples in which processing of pro-caspase-3 was evident (Fig. 5). Next, we examined caspase-6 using a caspase-6 antibody, which recognizes the full-length form and active p20 subunit of caspase-6. Immunoblot analysis revealed that the reduction of the band corresponding to the caspase-6 pro-form occurred as early as 24 hours. Furthermore, a band at approximately p20, corresponding to the carboxyl-terminal portion of caspase-6, was only easily detected in cell lysates after a 24-hour exposure to hypoxia (Fig. 6). Lamin A is believed to be an

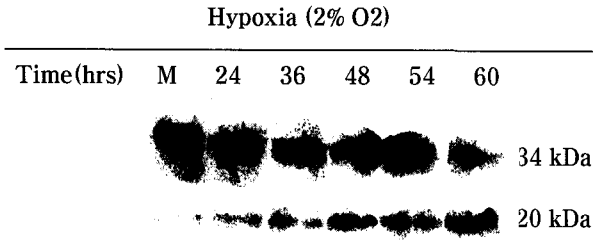

Fig. 6. Hypoxia induced caspase-6 proteolytic cleavage in MC3T3E1 osteoblasts.
MC3T3E1 cells were incubated for various periods in hypoxia environment. Total cell lysates were prepared and then subjected to western blot analysis as described in Materials and Methods using antibody for 17 kDa protein, active product of 20 kDa, active product of caspase-6.

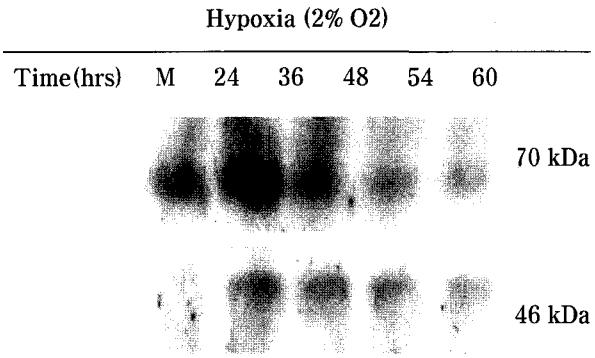

Fig. 7. Hypoxia induced Lamin A cleavage in MC3T3E1 osteoblasts.
MC3T3E1 cells were incubated for various periods in hypoxia environment. Total cell lysates were prepared and then subjected to western blot analysis as described in Materials and Methods using antibody for Lamin A.

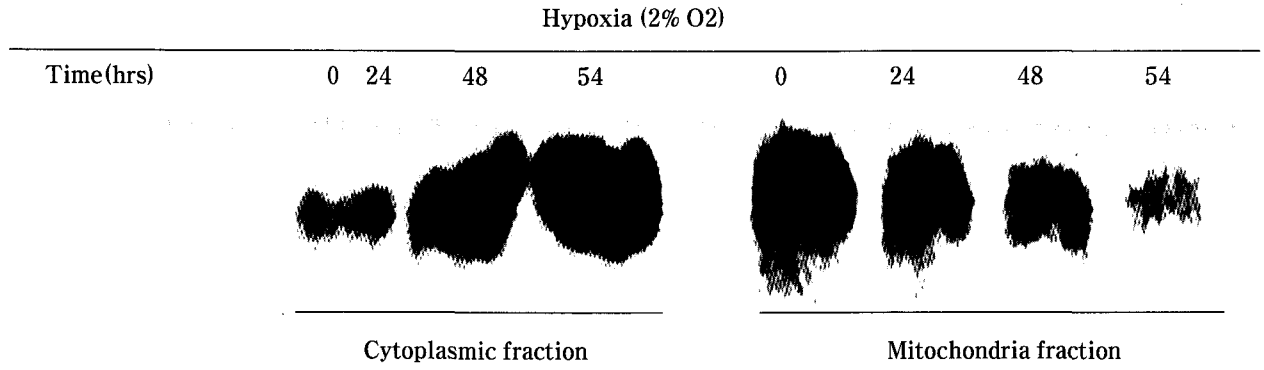

Fig. 8. Hypoxia induced cytochrome c release into the cytosol in MC3T3E1 osteoblasts.
MC3T3E1 cells were challenged with hypoxia for various periods. The mitochondrial and cytosol fractions were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Cytochrome c was detected by western blot analysis using a monoclonal antibody against cytochrome c.

endogeneous substrate for caspase-6. To ascertain whether apoptosis induced by hypoxia in the osteoblasts was accompanied by the activation of caspase-6, we examined the cleavage of Lamin A. As shown in Fig. 7, treatment with hypoxia resulted in Lamin A cleavage, yielding a 46-kDa fragment typical of caspase activity, in a time-dependent manner. The 46-kDa Lamin A fragment, which is indicative of proteolytic holoenzyme digestion, is visible after a 24hours incubation period with hypoxia.

3. Cytochrome C was released from the mitochondria during hypoxia-induced apoptosis

Many reporters suggested that cytochrome c could induce apoptosis in carrot cytosol and the stereotypical DNA ladder could be observed.[6] Cytochrome c has been able to be used as an apoptosis inducer only recently[16]. The steps involved in hypoxia signaling through cytochrome c have not been previously investigated in osteoblasts. Therefore, the amounts of cytochrome c in

the mitochondrial and cytoplasmic fractions were measured by western blot analysis. Without a hypoxia challenge, most of detectable cytochrome c was found in the mitochondrial fraction. Thus, the amount of cytochrome c in the cytosol fraction increased significantly after a 24-hour challenge with hypoxia, and the level continued to increase for up to 54 hours. The amount of cytochrome c in the mitochondrial fraction showed a corresponding decrease in a time-dependent manner. Therefore, the hypoxia-induced apoptosis was associated with a cytochrome c release into the cytoplasm in MC3T3E1 osteoblasts (Fig. 8).

DISCUSSION

The aim of this study was to explain the mechanism of hypoxia-induced cell death in MC3T3E1 osteoblasts. It is held that 2-5% O_2 corresponds to a physiologic hypoxia.[2,23] In this study, we showed by studying internucleosomal DNA fragmentation, and chromatin condensation we showed that exposure to physiologic hypoxia (2% O_2) elicited apoptotic cell death in MC3T3E1 osteo-blasts. We demonstrated that caspase (at least caspase -3 and 6) were activated by hypoxia during apoptosis. Caspase activation and apoptosis triggered by hypoxia were completely inhibited by treatment with a specific caspase-3 inhibitor (DEVD-CHO) or a pan-caspase inhibitor (Z-VAD-FMK). Furthermore, hypoxia resulted in the release of cytochrome c into the cytosol from the mitochondria (Fig. 8). These findings suggest that activation of caspases accompanied by a cytochrome c release in response to hypoxia may contribute to apoptotic cell death in this cell line. Hypoxic conditions induced DNA fragmentation in a time-dependent manner in MC3T3E1 cells (Fig. 3). We examined whe-ther caspases, known to be death proteases, are involved in hypoxia-induced apoptosis. Treatment with a caspase inhibitory peptide (Z-VAD-FMK) completely blocked the formation of a hypoxia-induced apoptosis (Fig. 2B). In addition, a caspase-3-specific inhibitory peptide (Z-DEVD-CHO) abrogated the hypoxia-induced apoptosis in MC3T3E1 osteoblasts. Enzymatic activity of a caspase-3-like protease was definitely detected in the cytosolic extracts using a fluorescent peptide substrate (DEVD-CHO), following treatment with hypoxia (Fig. 4). This suggested that hypoxia-induced apoptotic cell death through caspase activation.

Moreover, we found that caspase-3, 6, and Lamin-A also degrade during the apoptotic process induced by hypoxia in MC3T3E1 osteoblasts.

Caspase-6 has been shown to maintain the integrity of the nuclear envelope and cellular morphology. This indicates that caspase-6 may be activated by the apoptotic stimuli. We have shown that pro-caspase-6 was cleaved in response to hypoxia in MC3T3E1 cells. Recently, it has been revealed that the release of apop-togenic proteins, such as cytochrome c and apoptosis-inducing factor (AIF) from the mitochondria to the cytosol is involved in protease activation linked to apoptosis.[24] Mitochondrial damage has long been considered to play a role in hypoxic and ischemic cell death, but the contribution of apoptogenic mechanisms to these ubiquitous pathophysiological processes is poorly defined. Caspase inhibitors afford some protec-tion against ischemic injury, but it is not known whether they protect by decreasing inflammation, or by retarding hypoxia related death cascades in the parenchymal cells. This is an important consideration for mammalian cells, since they are dependent on mitochon-drial function for long-term viability. Cytochrome c is an essential component of the mitochondrial respiratory chain and is also released from the mitochondria in response to various stimuli that also lead to apoptosis. These include UV irradiation, etoposide, staurosporine, actinomycin D, H2O2 and Ara-C.[17,25] Zhivotovsky et al.[26] showed that injected cytochrome c induces apop-tosis in several different types of cells. Furthermore, it has been demonstrated that only the oxidized form of cytochrome c is able to activate caspases.[27] More recently, it was reported that cytosolic cytochrome c activates caspase-9, and subsequently activated caspase-9 cleaves and activates caspase-3.[28] In this

released into the cytosol in response to hypoxia. Western blot anaysis showed that significant amounts of cytochrome c are released 24 hours after a hypoxia challenge, and the amount continues to increase for up to 54 hours. The amount of cytochrome c in the mitochondrial fraction showed a crresponding decrease in a time-dependent manner. The appearance of cytochrome c was related to the activation of caspase-3-like protease (DEVDase), and a decrease in the amount of the proform of caspase-3 and 6.

These findings were of potential clinical significance. Our *in vitro* results suggested that therapies targeted at the inhibition of caspase-3-like protease activation by hypoxia should abrogate apoptosis in osteoblasts.

CONCLUSION

Mammalian cell function is dependent on a continuous supply of oxygen. Even brief periods of oxygen deprivation can result in profound cellular damage. The aim of this study was to examine the possible mechanism of apoptosis in response to hypoxia in MC3T3E1 osteoblasts.

MC3T3E1 osteoblasts under hypoxic conditions (2% oxygen) resulted in apoptosis in a time-dependent manner, determined by DNA fragmentation assay and nuclear morphology stained with fluorescent dye (Hoechst 33258). Pretreatment with Z-VAD-FMK (a pan-caspase inhibitor) or Z-DEVD-CHO (a specific caspase-3 inhibitor) suppressed the DNA ladder in response to hypoxia in a concentration-dependent manner. An increase in caspase-3-like protease (DEVDase) activity was observed during apoptosis, but no caspase-1 activity (YVADase) was detected. To confirm what caspases were involved in apoptosis, western blot analysis was performed using an anti-caspase-3 or 6 antibodies. The 17-kDa protein that corresponds to the active products of caspase-3 and the 20-kDa protein of the active protein of caspase-6 were generated in hypoxia-challenged lysates in which the full length forms of caspase-3 and 6 to evident. With a time course similar to caspase-3 and 6 activation, hypoxic stress also caused the cleavage of Lamin A, typical of caspase-6 activity. In addition, the hypoxic stress elicited the release of cytochrome c into the cytosol during apoptosis. These findings suggested that activation of caspases accompanied by a cytochrome c release in response to hypoxia is involved in apoptotic cell death in MC3T3E1 osteoblasts.

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국문초록

저산소 상태에서 조골세포 고사의 신호전달 기전

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본 연구는 MC3T3E1 조골세포가 저산소증에 반응하여 유발될 수 있는 세포 고사조절 기전을 구명하고자 함에 목적이 있다.

2% 저산소증의 조건하에서 MC3T3E1 조골세포는 DNA 사다리 분절 형성을 보였으며 형광성 염료인 Hoechst 33258로 염색된 핵 구조 형태 관찰시 시간이 지남에 따라 세포고사 현상을 관찰할 수 있었다. Pancaspase 억제제인 Z-VAD-FMK나 특정한 caspase-3 억제제인 Z-DEVD-CHO로 사전 처치하였을 경우에는 저산소증에 의한 DNA 사다리 분절 형성이 농축에 비례하여 억제되었다. caspase-3류의 프로테아제(DEVDase) 활성 증가가 세포고사 중에 관찰되었으나 caspase-1 (YVADase)의 활성은 없었다.

어떤 caspase가 세포고사에 관여하는지를 확인하기 위하여 anti-caspase-3 또는 anti-caspase-6의 항체를 이용한 western blotting이 시행되었다. caspase-3의 활성산물에 해당하는 17-KDa 단백질과 caspase-6의 활성산물인 20-

KDa 단백질이 세포용해물에서 발생되었다.

또한 시간 경과와 더불어 caspase-6의 활동의 상징인 Lamin A의 분열을 일으켰으며, 사이토크롬 C를 cytosol로 방출하였다.

이로써 저산소증에 의한 조골세포의 고사 과정에 사이토크롬 C의 방출이 포함된 caspase의 활성이 관여한다는 것을 확인할 수 있었다.

주요 단어 : 저산소, 조골세포, 고사