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Role of p-38 MAP Kinase in apoptosis of hypoxia-induced osteoblasts

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Tooth movement by orthodontic force effects great tissue changes within the periodontium, especially by shifting the blood flow in the pressure side and resulting in a hypoxic state of low oxygen tension.

The aim of this study is to elucidate the possible mechanism of apoptosis in response to hypoxia in MC3T3E1 osteoblasts, the main cells in bone remodeling during orthodontic tooth movement.

MC3T3E1 osteoblasts under hypoxic conditions (2% oxygen) resulted in apoptosis in a time-dependent manner as estimated 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, completely suppressed the DNA ladder in response to hypoxia. 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 are involved in apoptosis, Western blot analysis was performed using anti-caspase-3 or -6 antibodies. The 10-kDa protein, corresponding to the active products of caspase-3, and the 10-kDa protein of the active protein of caspase-6 were generated in hypoxia-challenged cells in which the processing of the full length form of caspase-3 and -6 was evident. While a time course similar to this caspase-3 and -6 activation was evident, hypoxic stress caused the cleavage of lamin A, which was typical of caspase-6 activity. In addition, the stress elicited the release of cytochrome c into the cytosol during apoptosis. Furthermore, we observed that pre-treatment with SB203580, a selective p38 mitogen activated protein kinase inhibitor, attenuated the hypoxia-induced apoptosis. The addition of SB203580 suppressed caspase-3 and -6-like protease activity by hypoxia up to 50%. In contrast, PD98059 had no effect on the hypoxia-induced apoptosis. To confirm the involvement of MAP kinase, JNK/SAPK, ERK, or p38 kinase assay was performed. Although p38 MAPK was activated in response to hypoxic treatment, the other MAPK -JNK/SAPK or ERK- was either only modestly activated or not at all.

These results suggest that p38 MAPK is involved in hypoxia-induced apoptosis in MC3T3E1 osteoblasts.

Key words : Hypoxia, Apoptosis, Osteoblast , P-38 MAP kinase

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Tooth movement by orthodontic force effects great tissue changes within the periodontium, especially by shifting the blood flow in the pressure side and resulting in a hypoxic state of low oxygen tension. Hypoxia could lead to apoptosis in osteoblasts, the main cells in bone remodeling during orthodontic tooth movement.

Apoptosis plays an important role in both physiological and pathological conditions. In the development of the embryo, an overproduction of cells ultimately requires that surplus elements die as part of the process that balances growth and differentiation with death; thus, insight into apoptosis mechanisms will contribute to the improved understanding and treatment of diseases in which the normal balance between cell proliferation and cell death is disrupted.[1-3]

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] 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] 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.[7] These effector caspases then cleave into the intracellular substrates, such as poly (ADP-ribose) polymerase (PARP) and lamins, during the execution phase of apoptosis.[8] As caspase-8 can activate all known caspases *in vitro*, it is a prime candidate for an initiator caspase in many forms of apoptosis.[9] 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.[10,11] Active caspase-9 can then catalyze the effectors caspase-3, -6, and 7.[12] 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.[13] Release of mitochondrial cytochrome c and activation of caspase-3 are blocked by anti-apoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-XL, and promoted by pro-apoptotic members such as Bax and Bak.[14,15]

One of the important signaling mediators in caspase activation and cytchrome c translocation can be mitogen activated protein kinase (MAPK) activation. Among MAPK subfamilies, a potent signaling candidate is p38 MAPK known as stress-related protein kinase. The p38 MAPK has been reported as being involved in the redox-related signaling pathways that lead to apoptosis.[16] In addition, the stress- and mitogen- activated protein kinase (SAPK and MAPK) pathways can play a critical role in caspase activation and resultant apoptosis.[16,17] These three MAPKs, extracellular -related kinase (ERK) 1/2, c-Jun N-terminal kinase (JNK), and p38, are known to be activated upon the dual phosphorylation of Thr or Tyr. Substrates of MAPKs such as transcription factors Elk-1, c-Jun, and ATF-1 are then phosphorylated and activated, which results in the transcription of specific genes.[18-20] The mechanism by which a specific MAPK responds to the input of distinct stress-induced signal transduction pathways and the resulting biological functions of each pathway have been investigated extensively.[21] We therefore investigated the effect of hypoxia on MAPK signaling pathways in MC3T3E1 osteoblasts.

In this study, SB203580, a specific p38 MAPK inhibitor, significantly reduces hypoxia-stimulated apoptotic pathways including caspase-3 or -6 activity, and cytochrome c release. We therefore suggest that p38 MAPK activation might be responsible for hypoxia-induced apoptosis in MC3T3E1 osteoblasts.

MATERIALS AND METHODS

Materials

Anti-p38, anti-JNK1, anti-ERK1, caspase-3,

caspase-6 p10, lamin A, cytochrome c antibody, GST-ATF2, and myelin basic protein (MBP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). GST-c-Jun N-terminal protein was purchased from Stratagene (La Jolla, CA, U.S.A.). Hoechst 33258, diphenylamine and 3-[4,5-dime-thylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). PD98059 (2-(2-amino-methoxyphenyl)-oxanaphalen-4-one) and SB203580 were obtained from New England Biolabs (Beverly, MA, U.S.A.). N-acetylAsp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC), Ac-YVAD-AMC, and Pansorbin were obtained from Calbiochem-Behring Corp. (La Jolla, CA, U.S.A.). Z-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK) was purchased from Kamiya Bio Company (Seattle, WA, U.S.A.). A genomic DNA purification kit was obtained from Promega (Promega Co, Wisconsin Medicine, WI, U.S.A.). All cell culture media and reagents were obtained from Life Technologies, Inc. (Ca., U.S.A.).

Cell culture and hypoxic treatment

The clonal murine osteoblast cell line, MC3T3E1, was cultured in -modified essential medium (-MEM) (GIBCO BRL Co., New York, NY, U.S.A.), and supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. Our system for exposing cell cultures to hypoxia creates defined atmospheric oxygen partial pressure (pO_2) values within the range of approximately 2%. We define the low oxygen conditions used for these studies as hypoxia, not anoxia. When MC3T3E1 osteoblasts achieved 80% confluence, the hypoxic experiments were all performed at pO_2=2% in a mixture of 5% CO_2 and the balance N2 in a humidified 37℃ incubator (ANX-1, Hirasawa, Tokyo, Japan). Atmospheric oxygen levels in this apparatus were calibrated by using a polarographic oxygen electrode (Oxygen Sensors, Inc., Norriston, PA, U.S.A.).

MTT assay

The MTT assay of cell viability was performed as described previously.[22] Briefly, after exposure to hypoxia, MTT was added to cells for a final concentration of 0.5 mg/ml MTT reagent and incubated at 37℃ for 5 hours. The medium was aspirated and the formazan product was solubilzed with dimethylsulfoxide. Absorbance at 630 nm (backward absorbance) was subtracted from absorbance at 570 nm for each well.

Agarose gel electrophoresis for DNA fragmentation

The characteristic ladder pattern of DNA break was analyzed by agarose gel electrophoresis. Briefly, DNA from the MC3T3E1 cells (1×10^6 cells/each group) was isolated by a Wizard Genomic DNA purification kit and isolated by serial ethanol precipitation. Isolated genomic DNA (10 ㎍) was subjected on 1.5% of agarose electrophoresis at 100 V for 1 hour. DNA was visualized by staining with ethidium bromide under UV light.

Morphological detection of apoptosis

Morphological evaluation of apoptotic cell death was performed as previously described with some modification.[23] Coverslips were fixed for 5 minutes in 3 % paraformaldehyde in phosphate-buffered saline. After air-drying, coverslips were stained for 10 minutes in Hoechst 33258 (10 ㎍/ml), mounted in 50% glycerol containing 20 mM citric acid and 50 mM orthopho-sphate, and stored at -20℃ before analysis. Nuclear morphology was evaluated using a Zeiss IM 35 fluorescent microscope at an excitation and emission wavelength of 440 and 460 nm, respectively. Apoptotic cells were identified as those whose nucleus exhibited brightly staining condensed chromatin or nuclear fragmentation.

Quantitation of DNA fragmentation

DNA fragmentation was essentially assayed as

previously reported.[24] Briefly, after incubation, cells were scrappered off the culture plates, resuspended in 250 μl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and incubated with an additional volume lysis-buffer (5 mM Tris, 20 mM EDTA, 0.5% Triton X-100, pH 8.0) for 30 minutes at 4℃. Samples were pelleted at 4000 rpm for 10 min and the supernatant was removed. Intact chromatin (pellet) was separated from DNA fragments (supernatant) by centrifugation. After addition of 300 μl 5% trichloroacetic acid, samples were boiled for 15 minutes. DNA contents were quantitated using the diphenylamine reagent. The percentage of fragmented DNA was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet.

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 g/ml leupeptin, aprotinin (0.27 trypsin inhibitor/U/ml), and 100 M PMSF. Caspase-1 or caspase-3-like activity was determined by the incubation of cell lysate (containing 25 μg of total protein) with 50 μM of the fluorogenic substrate, AC-YVAD-AMC or AC-DEVD-AMC, respectively, in a 200 l cell-free system buffer. The buffer comprises 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.

Cytochrome c measurements

Mitochondrial fractions were prepared from 1×10^7 MC3T3E1 cells by differential centrifugation in a buffer containing 250 mM sucrose as previously described.[1] Protein samples of 25 μg were loaded on sodium dodecyl sulfate-15% polyacrylamide gels (SDS-PAGE), subjected to electrophoresis, and then electrophoretically transferred to nitrocellulose membranes. Western blots were probed with primary monoclonal anti-cytochrome c antibody and secondary anti-mouse horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology) and then developed with enhanced chemiluminescence (Amersham Life Science).

Immunoprecipitation and kinase assay

Cells were lysed in a modified radioimmune precipitation buffer (25 mM Tris-HCl, pH 8.0, containing 137 mM NaCl, 10% (v/v) glycerol, 0.1% SDS, 0.5% (v/v) deoxycholate, 1% (v/v) Nonidet p-40, 2 mM EDTA, 1 mM pefabloc, 1 mM sodium vanadate, 5 mM benzamidine, 5 μg/ml aprotinin, and 5 μg/ml leupeptin) on ice for 30 minutes. The cell debris was removed by centrifugation at 15,000 rpm for 10 minutes. The supernatants were then incubated with anti-p38 for 2 hour at 4℃. The immunocomplexes were precipitated with Pansorbin (Calbiochem, Ca, U.S.A.) and washed extensively with the lysis buffer (50 mM LiCl/100 mM, Tris-HCl (pH 7.6)/0.1% (v/v) Triton X-100/1 mM DTT). The pellets were left as a 1 : 1 suspension in the assay buffer and 20 μl (0.3 mg/ml) of GST-c-Jun (JNK/SAPK), MBP (ERK1), or GST-ATF-1 (p38 MAPK) was added. Kinase reactions were initiated by the addition of 15 l of γ^{32}P-labelled Mg/ATP solution (50 mM $MgCl_2$/500 μM ATP/10 μCi of [γ-^{32}ATP]) and performed at 30℃ for 30 minutes. The reactions were stopped by the addition of Laemmli sample buffer and boiling for 5 minutes. The samples were separated by SDS/PAGE (12% (w/v) gel) and were subjected to autoradiography after drying. Quantification was performed with a phosphoimager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). MAPK activity assays were performed essentially as described previously.[25]

Data presentation and statistical analysis and kinase assay

Data are presented as means×S.D. Student's t-test was used for statistical analysis. Results were considered statistically different at the 5% significance level.

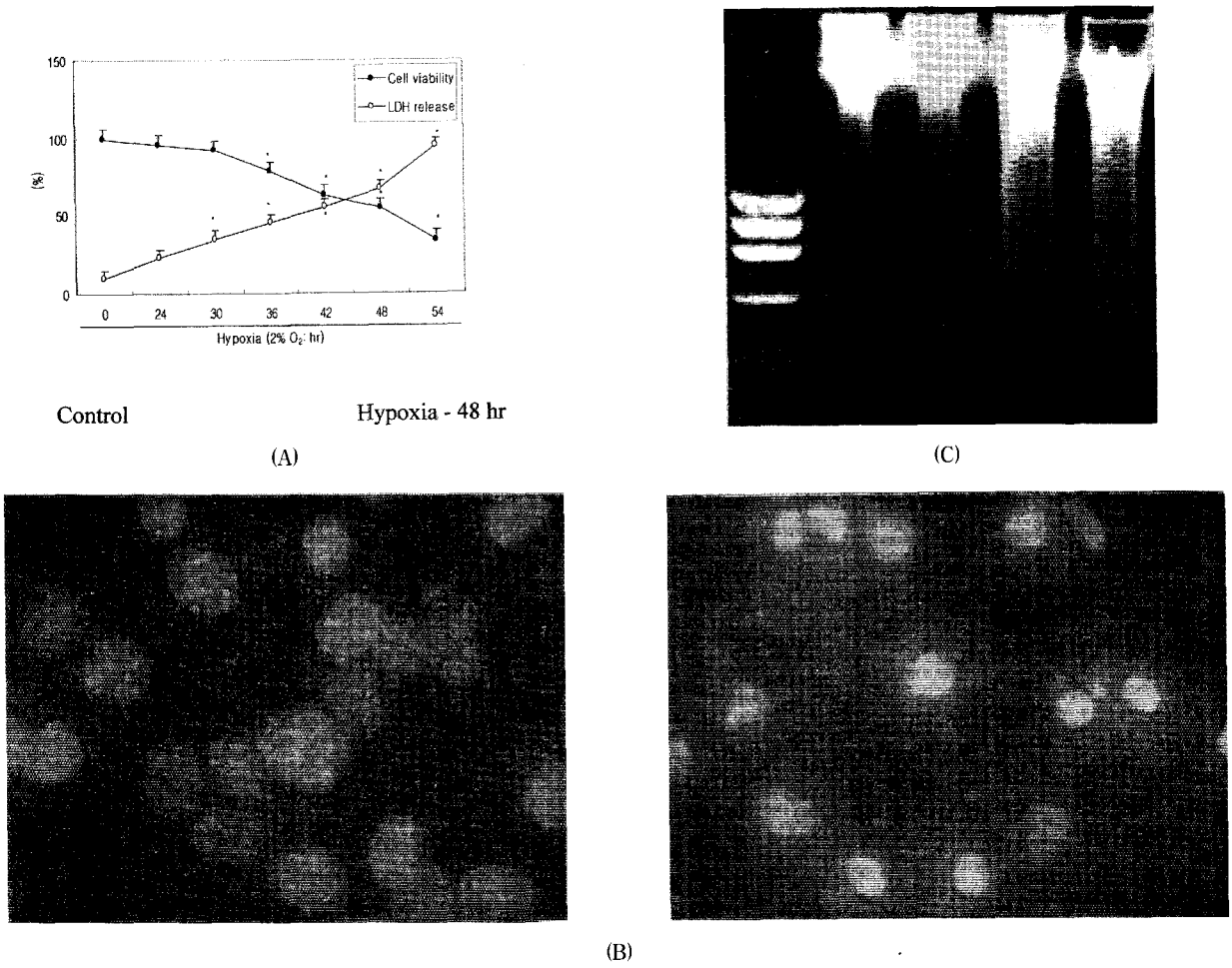


Fig. 1. Hypoxia induces apoptosis in MC3T3E1 osteoblasts. Cell death was assessed by MTT assay, morphology and DNA fragmentation. MC3T3E1 were subjected to hypoxia (0, 24, 30, 36, 42, or 48 hr) in full culture medium. (A) MTT cell viability and LDH release assay were performed in a various time point. Data are expressed as the percent of controls that were not exposed to hypoxia. Means±S.D. (n=4). *Significantly different from control, P0.05. (B) Cells were stained with Hoechst 33258. (C) Cells were then harvested and assayed for DNA fragmentation.

RESULTS

Hypoxia induces apoptotic cell death in MC3T3E1 cells

To address the ability of hypoxia to induce cell death, we first investigated the effect of hypoxia on cell viability using MTT assay and LDH release. 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. 48 hours later, a loss of viability occurred following hypoxia in a time-dependent manner (Fig. 1A). 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 Hoechst dye 33258 were examined (Fig. 1B). A major component of hypoxia-induced cell death was attributable to apoptosis, as shown by DNA laddering. Significant DNA laddering was present after 48 hours of

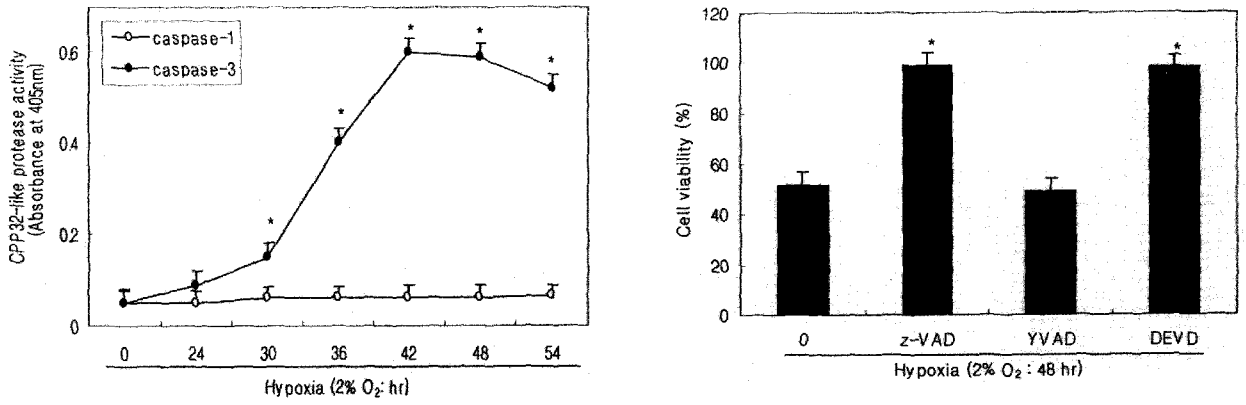


Fig. 2. MC3T3E1 cell death in hypoxia is mediated by caspases. MC3T3E1 cells were exposed to hypoxia for various time points. (A) Cell extracts (50 μg) were then incubated in the presence of fluorescent caspase-3 substrate DEVD-AFC (25 μM) for 1 hr at 37℃. Caspase-3 activity was measured fluorometrically after substrate cleavage with excitation at 400 nm and emission at 505 nm. (B) MTT assay was performed after 48 hr of exposure to hypoxia with or without pretreatment with pan-caspase inhibitor Z-VAD-FMK (100 μM), caspase-1 inhibitor YVAD-CHO (100 μM), or caspase-3 inhibitor DEVD-CHO (100 μM). Data are expressed as the percent of controls that were not exposed to hypoxia. Means±S.D. (n=4). *Significantly different from control, P0.05.

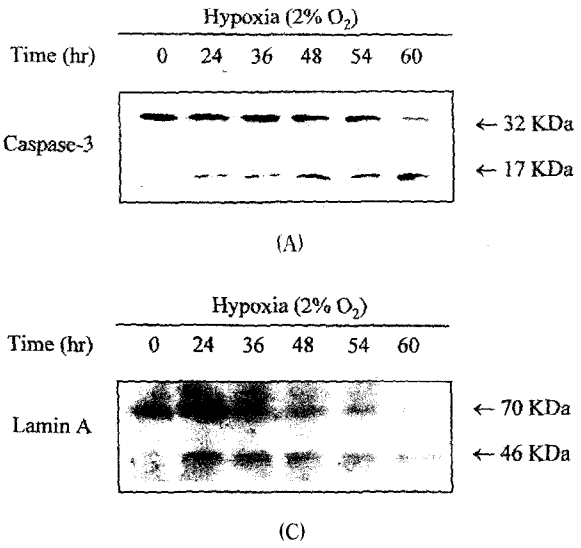
exposure of MC3T3E1 cells but was not seen in control at the same time point (Fig. 1C). In addition, apoptotic cells were identified by their condensed and fragmented nuclei. Approximately, 45% of cells were apoptotic when evaluated by Hoechst 33258 staining (data not shown).

Hypoxia induces apoptotic cell death via caspase activation

The caspase family of proteases is thought to be the final execution pathway in apoptosis.[22,26] Having established a role for caspases in cell death induced by hypoxia, we analyzed the process of caspase activation in this system. As shown in Fig. 2A, 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 apoptosis of osteoblasts, we treated MC3T3E1 cells with pan-caspase inhibitor Z-VAD-FMK, specific caspase-1 inhibitor, YVAD-CHO, or specific caspase-3 inhibitor, DEVD-CHO. Fig. 2B shows that pretreatment of MC3T3E1 cells with 100 M Z-VAD-FMK or 100 μM DEVD-CHO largely prevented the cell death seen after 48 hours of hypoxia. The formation of a DNA ladder was also suppressed by simultaneous treatment with 100 μM Z-DEVD-CHO or Z-VAD-FMK (data not shown).

Caspase-3 and -6 are synthesized as precursor molecules, and are approximately 32-kDa and 34-kDa in size, respectively.[27] During processing, caspase-3 and -6 are proteolytically cleaved to produce a mature enzyme composed of each 17 and 20-kDa subunit, respectively.

As shown in Fig. 3A, new bands corresponding to p17 of caspase-3 were detected at 24 hours after hypoxic challenge in samples in which processing of pro-caspase-3 was evident (Fig. 3A). Next, we examined caspase-6 using caspase-6 antibody, which recognizes the full-length form and active p20 subunit of caspase-6. Immunoblot analysis revealed that reduction of the band corresponding to the caspase-6 pro-form occurred and a band at approximately p20, corresponding to the carboxyl-terminal portion of caspase-6, was easily detected in cell lysates after a 24-hour exposure to hypoxia (Fig. 3B). And then we



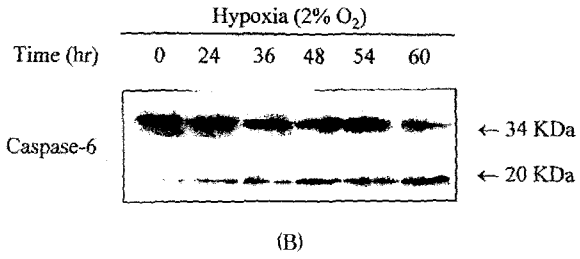


Fig. 3. Decrease in pro-forms of caspase-3 and -6, and lamin A in response to hypoxia in MC3T3E1 cells. The cells were incubated for various periods in hypoxic environment. Total cell lysates were prepared and then subjected to western blot analysis using specific antibodies for caspase-3 (A), -6 (B), and lamin A (C). These are typical results from three independent experiments.

investigated the cleavage of lamin A by treatment with hypoxia in MC3T3E1 cells. Lamin A, which is believed to be an endogeneous substrate for caspase-6, is critical to maintaining the integrity of the nuclear envelope and cellular morphology. To ascertain whether apoptosis induced by hypoxia in the osteoblasts is accompanied by the activation of caspase-6, we examined the cleavage of lamin A. As shown in Fig. 3C, treatment with hypoxia resulted in lamin A cleavage, yielding a 46-kDa fragment which was typical of caspase-6 activity, in a time-dependent manner. The 46-kDa lamin A fragment, which is indicative of proteolytic holoenzyme digestion, was visible after a 24-hour incubation period with hypoxia. The lamin A cleavage coincided with the appearance of DNA fragmentation (Fig. 1B).

Cytochrome c is 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 used as an apoptosis inducer only recently.[13] The steps involved in hypoxia signaling through cytochrome c have not been previously investigated in osteoblasts. Therefore the amounts of cytochrome c in the mitochondria and

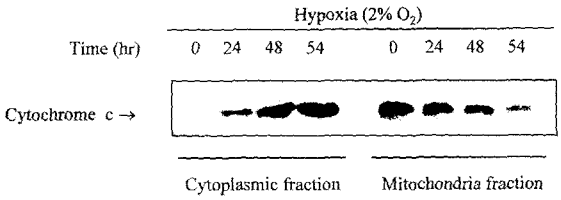


Fig. 4. Cytochrome c release into the cytosol by hypoxia. The cells were challenged with hypoxia for various periods. The cytosol fractions were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Cytochrome c was detected by western blot analysis using a monoclonal antibody against cytochrome c. These are typical results from three independent experiments.

the amounts of cytochrome c in the mitochondria and cytosol fractions were then measured by Western blot analysis. Without hypoxia challenge, most of the detectable cytochrome c was found in the membrane fraction. Thus, the amount of cytochrome c in the cytosol fraction increased significantly after a 48-hour challenge with hypoxia, and the level continued to increase for up to 54 hours. The amounts of cytochrome c in the membrane fraction showed a corresponding decrease in a time-dependent manner. Therefore, the hypoxia-induced apoptosis is associated with cytochrome c release into the cytosol in MC3T3E1 osteoblasts (Fig. 4).

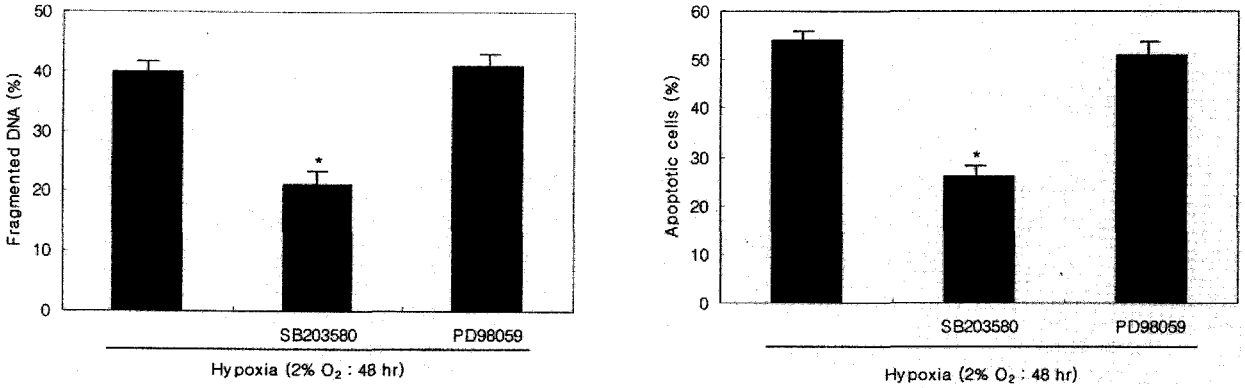


Fig. 5. Effect of a specific p38 inhibitor, SB203580 or ERK inhibitor, PD098059 on hypoxia-induced apoptosis in MC3T3E1 osteoblasts. MC3T3E1 cells pretreated with SB203580 (20 μM) or PD98059 (20 μM) were exposed to hypoxia for 48 hr. (A) DNA fragmentation assay was performed with diphenylamine reagent. (B) The fixed cells were incubated with Hoechst 33258 (2.5 μg/ml in PBS). The nucleus condensation of the cells was analyzed with a fluorescence microscope. Apoptotic cells were quantified by fluorescence microscopy. Data are expressed as the percent of controls that were exposed to hypoxia. Means±S.D. (n=4). *Significantly different from control, P0.05.

SB203580, a p38 inhibitor, partially inhibits hypoxia-induced apoptosis in MC3T3E1 cells

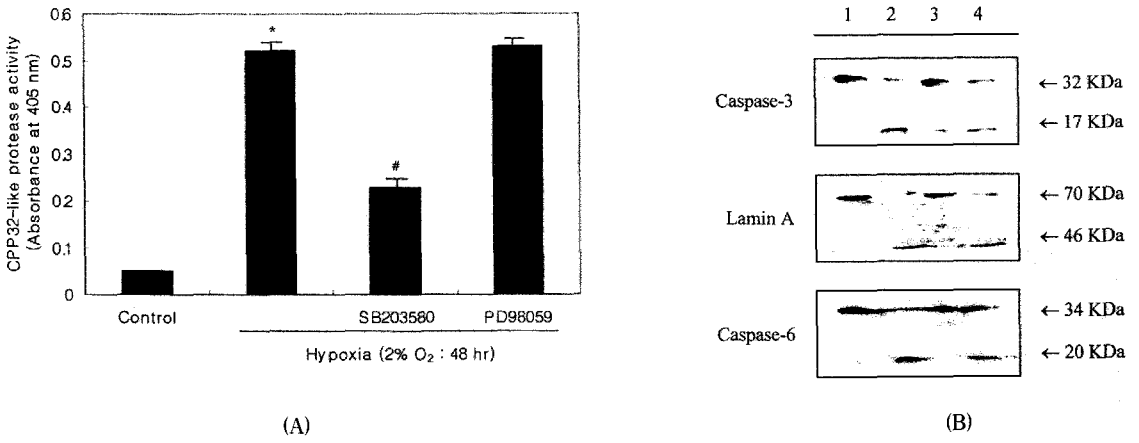
To further assess the role of MAPK in hypoxia-induced apoptosis, PD98059, a cell permeable MEK inhibitor, or SB203580, the specific inhibitor for p38 MAPK activity28) was used to pharmacologically block MAPK activation. MC3T3E1 osteoblasts were pretreated with 20 M PD098059 or 20 M SB203580 for 30 minutes prior to the exposure of hypoxia. And then the effects of the inhibitors on hypoxia-induced apoptosis were analyzed by DNA fragmentation assay (Fig. 5A) and quantification of apoptotic cells with fluorescence microscopy (Fig. 5B). It is evident that SB203580, but not PD98059, repressed hypoxia-induced apoptosis in MC3T3E1 osteoblasts up to 50%, indicating that p38 MAPK may be related to hypoxia-induced apoptosis in MC3T3E1 osteoblasts.

SB203580, a p38 inhibitor, inhibits hypoxia-induced caspase activation in MC3T3E1 cells

We also observed that the moderately inhibitory effect on apoptosis by SB203580 was associated with suppressed caspase-3-like activity. As shown in Fig. 6A, SB203580, a specific p38 inhibitor, significantly repressed the hypoxia-induced caspase-3-like protease activation. For SB203580, but not PD98059-treated cells, the activity of caspase-3 was reduced to 45%. These results indicate that only p38 MAPK, but not ERK is involved in hypoxia-stimulated caspase-3 followed by apoptosis in MC3T3E1 osteoblasts. Having demonstrated that SB203580, a specific p38 inhibitor, inhibits the hypoxia-induced activation of caspase in the osteoblasts, then we investigated the inhibitory effect of proteolytical cleavage of caspase-3 and -6 proteins by SB203580. As shown in Fig. 6B, the inhibitor reduced the hypoxia-induced cleavage of caspase-3, -6, and lamin A significantly. Our results show that p38 MAPK plays an inhibitory effect on hypoxia-induced caspase activation followed by apoptosis in the osteoblasts.

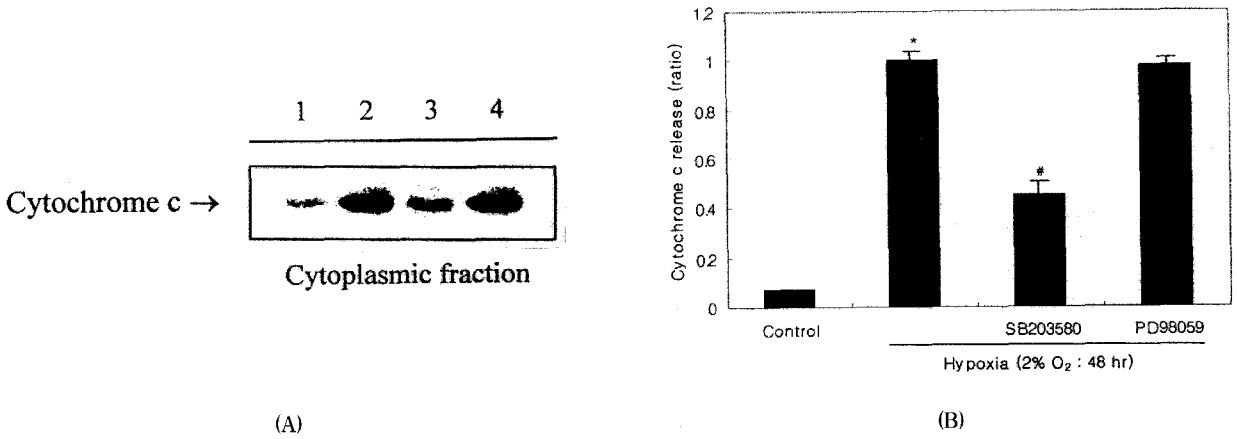
SB203580 has an inhibitory effect on hypoxia-induced cytochrome c in MC3T3E1 cells

Next we observed that the pretreatment of cells with



(A) (B)

Fig. 6. Effect of SB203580 on hypoxia-induced caspase-3 activity in MC3T3E1 osteoblasts. The cells were exposed to hypoxia for 48 hr with or without 20 μM SB203580. (A) And then caspase-3-like activity was measured as described under Materials and Methods. Data are expressed as the percent of controls that were not exposed or exposed to hypoxia. Means±S.D. (n=4). *Significantly different from control, P0.05. #Significantly different from hypoxic control, P0.05. (B) Total cell lysates were prepared and then subjected to western blot analysis using specific antibodies for caspase-3, -6 and lamin A. These are typical results from three independent experiments. 1: Control, 2: Hypoxia (48 hr), 3: SB203580 (20 μM) + Hypoxia (48 hr), 4: PD98059 (20 μM) + Hypoxia (48 hr).



(A) (B)

Fig. 7. Effect of SB203580 on hypoxia-induced cytochrome c release in MC3T3E1 osteoblasts. Mitochondrial and cytoplasmic fractions were prepared and separated on SDS-PAGE and transferred onto a nitrocellulose membrane. (A) Cytochrome c was visualized by western blot analysis. 1: Control, 2: Hypoxia (48 hr), 3: SB203580 (20 μM) + Hypoxia (48 hr), 4: PD98059 (20 μM) + Hypoxia (48 hr). (B) These results are expressed quantitatively.

SB203580, a relatively selective inhibitor of p38, was able to reverse in part the down regulation of cytochrome c by hypoxia in a dose-dependent manner (Fig. 7A). These results are expressed quantitatively in Fig. 7B, where it can be seen that pretreatment with SB203580 resulted in a partial, but statistically significant, recovery of cytochrome c in mitochondria. The fact that SB203580 partially reversed the effects of hypoxia may be because this drug regulates hypoxia-induced cytochrome c release to the cytoplasm.

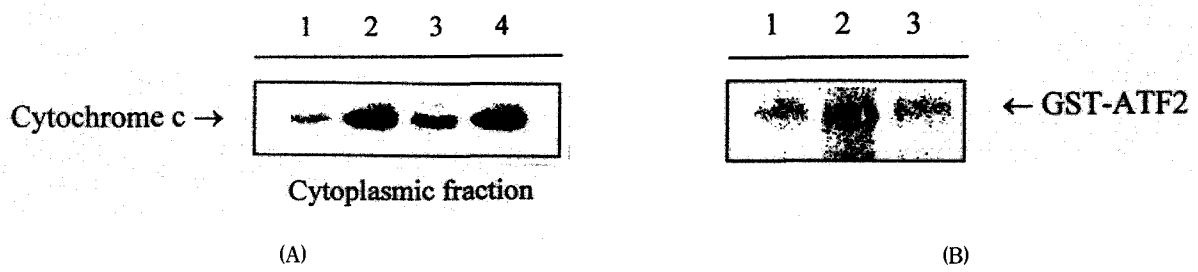


Fig. 8. Effect of hypoxia on p38 MAPK activation in MC3T3E1 osteoblasts. (A) MC3T3E1 cells were exposed to hypoxia for various times between 0 and 8 hr, as indicated. (B) MC3T3E1 cells were exposed to hypoxia in the presence or absence of SB203580 (20 μM) for 2 hr. Endogenous p38 MAP kinase activity was examined by immunocomplex assays as described in Materials and Methods. These are typical results from three independent experiments. 1: Control, 2: Hypoxia (2 hr), 3: Hypoxia (2 hr) + SB203580 (20 μM).

Hypoxia activates p38 MAPK in MC3T3E1 osteoblasts

Previously, investigators have shown that the p38 MAPK pathway is involved in and required for ischemia or ischemia/reperfusion-induced apoptosis in various cell types.[29,30] In this study, we found that hypoxia stimulated the phospho-transferase activity of p38 MAPK toward ATF-2 protein at 2 and 4 hours (4 fold). This transient increase of p38 MAPK activity was returned to the basal level around 4 hours after exposure of hypoxia (Fig. 8A). We then tested if the pre-treatment of SB203580, a specific p38 MAPK inhibitor, had a suppressive effect on hypoxia-induced p38 MAPK activation in MC3T3E1 osteoblasts. As shown in Fig. 8B, SB203580 (20 M) reduced hypoxia-stimulated p38 MAPK activity to the basal level. These results indicate that the activation of p38 MAPK may be related to hypoxia-induced apoptosis.

Hypoxia does not activate JNK/SAPK MAPK in MC3T3E1 osteoblasts

We next evaluated the effect of hypoxia on JNK, another SAPK. MC3T3E1 cells were exposed to hypoxia for various times, from 10 minutes to 8 hours, and JNK enzyme activity was measured in an immune complex kinase assay as described under Materials and Methods. Unlike its effects on p38 MAPK, hypoxia did not alter JNK enzyme activity significantly (Fig. 9). Our data shows that JNK/SAPK is not related with hypoxia-induced apoptosis in MC3T3E1 osteoblasts.

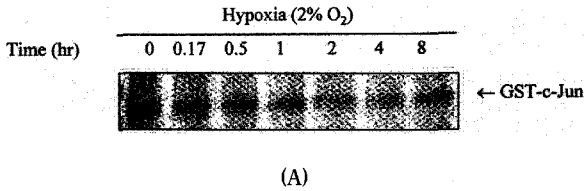
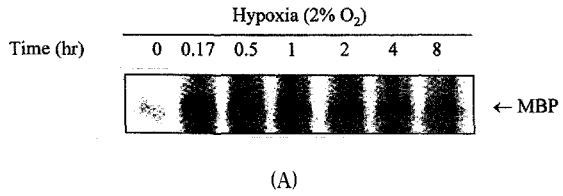


Fig. 9. Lack of effect of hypoxia on JNK/SAPK activity in MC3T3E1 osteoblasts. MC3T3E1cells were exposed to hypoxia for various times between 0 and 8 hr, as indicated. Endogenous JNK1 kinase activity was examined by immunocomplex assays as described in Materials and Methods. These are typical results from three independent experiments.

Hypoxia modestly activates ERK1 in MC3T3E1 osteoblasts

To determine the effect of hypoxia on ERK, MC3T3E1 cells were exposed to hypoxia for various times between 10 minutes and 8 hours. ERK1 enzyme activity was measured in an immunocomplex kinase assay. The MAPK pathway mediates the stimulation of cell



(A)

Fig. 10. Effect of hypoxia on ERK1 activity in MC3T3E1 osteoblasts. MC3T3E1cells were exposed to hypoxia for various times between 0 and 8 hr, as indicated. Endogenous ERK1 kinase activity was examined by immunocomplex assays as described in Materials and Methods. These are typical results from three independent experiments.

proliferation and has been suggested to be a negative regulator of apoptosis. The sustained activation of ERK with the stimuli may be related with signaling pathways other than the apoptotic signaling pathway. On the basis of the result that hypoxia-induced apoptosis was not inhibited by PD98059 (20 μM), ERK is not related with the apoptosis in MC3T3E1 osteoblasts (Fig. 10).

DISCUSSION

Cellular hypoxia is an important component of several pathophysiological conditions, including tumorigenesis and ischemia-related disorders. In these and other hypoxic situations mammalian cells alter gene expression to counter the effects of limited O_2. Hypoxia induces the transcriptional activation of a variety of genes, including erythropoietin, vascular endothelial growth factor, transferrin, tyrosine hydroxylase, and various glycolytic enzymes, all of whose products are involved in cellular adaptation to decreased O_2.[4]

Mammalian cell function is critically dependent on a continuous supply of oxygen. Even brief periods of oxygen deprivation can result in profound cellular and tissue damage. Thus, it is vital that organisms meet changes in O2 tension with appropriate cellular adaptations; however, the specific intracellular pathways by which this occurs are not well delineated. The stress- and mitogen-activated protein kinase (SAPK and MAPK) pathways play a critical role in responding to cellular stress and promoting cell growth and survival.31, 32) We therefore investigated the effect of hypoxia on the SAPK and MAPK signaling pathways.

SAPKs and MAPKs are the downstream components of three-member protein kinase modules[3]. Five homologus subfamilies of these kinases have been identified, and the three major families include p38/SAPK2/RK, JNK/SAPK, and p42/p44 MAPKs/ERKs.[33,34] In general, the stress-activated protein kinases (p38 and JNK) are activated primarily by noxious environmental stimuli such as ultraviolet light, osmotic stress, inflammatory cytokines, and the inhibition of protein synthesis.[35,36] However, increasing evidence suggests that, at least under certain conditions, these pathways can also be activated by mitogenic and neurotrophic factors.[37,38] In contrast, p42/p44 MAP kinases are stimulated primarily by mitogenic and differentiative factors in a Ras-dependent manner, although these enzymes can also be activated by certain environmental stressors.[31,32] Thus, we hypothesized that hypoxia, a prevalent physiological stressor in many disease states, may regulate the activity of the SAPK and MAPK signaling pathways.

The aim of this study was to elucidate the mechanism of hypoxia-induced cell death in MC3T3E1 osteoblasts. We showed by studying internucleosomal DNA fragmentation and chromatin condensation that exposure to hypoxia elicits apoptotic cell death. We demonstrated that caspases (at least caspase-3 and caspase-6) are activated by hypoxia during apoptosis (Figs. 2 and 3). Caspase activation and apoptosis triggered by hypoxia were completely inhibited by treatment with a specific caspase-3 inhibitor, DEVD-CHO or pan-caspase inhibitor, Z-VAD-FMK. Furthermore, hypoxia resulted in the release of cytochrome c into the cytosol from the mitochondria (Fig. 3). These findings suggest that activation of caspases accompanied by cytochrome c released in response to hypoxia may be involved in apoptotic cell death in this cell line. Hypoxic conditions induced DNA fragmentation in a time-dependent manner in MC3T3E1 cells (Fig. 1C).

We examined whether caspases, known to be death proteases, are involved in hypoxia-induced apoptosis. 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. 2C). This suggests that hypoxia induces apoptotic cell death through caspase activation. Furthermore, treatment with caspase inhibitory peptide (Z-VAD-FMK, a pan caspase inhibitor, or CHO-DEVD, a caspase-3 inhibitor) abrogated the hypoxia-induced apoptosis in MC3T3E1 osteoblasts (Fig. 2B). Moreover, we found that caspase-3, -6, and lamin A also degraded during the apoptotic process induced by hypoxia, with a time course similar to that of a DNA ladder formation.

Interestingly, SB203580, a specific p38 MAPK inhibitor, reduced the cytotoxity of hypoxia up to 50%, as shown in DNA fragmentation assay. Consistent with the result, SB203580 pretreatment partially but significantly inhibited hypoxia-induced caspase-3 activation in MC3T3E1 osteoblasts. Here we demonstrate conclusively that hypoxia elicits apoptosis partially in MC3T3E1 osteoblasts via caspase activation. Furthermore, p38 MAPK plays an important role in the apoptosis mechanism. In contrast, we could not detect caspase-1 activity in response to hypoxia using another fluorescent peptide substrate (YVAD-CHO) (Fig. 2A). Similar results in published reports found that caspase-1 activity is not observed during apoptosis accompanying treatment with thapsigargin[22] or cytokine deprivation.[39] This result indicates that hypoxia may activate a caspase-3-like protease through p38 MAPK activation.

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 showed evidence for the first time that pro-caspase-6 is cleaved in response to hypoxia and p38 is related to caspase-6 activation in MC3T3E1 cells. Recently, it has been revealed that the release of apoptogenic proteins, such as cytochrome c and apoptosis-inducing factor (AIF), from the mitochondria to the cytosol, is involved in protease activation linked to apoptosis.[40] 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.14) Zhivotovsky et al.[41] showed that injected cytochrome c induces apoptosis in several different types of cells. Furthermore, it has been demonstrated that only the oxidized form of cytochrome c is able to activate caspases.[42] More recently, it was reported that cytosolic cytochrome c activates caspase-9, and subsequently activated caspase-9 cleaves and activates caspase-3.[10] In this osteoblast cells, we examined whether cytochrome c is released into the cytosol in response to hypoxia. Western blot analysis showed that significant amounts of cytochrome c is released at 24 hours after a challenge with hypoxia, and the amount continues to increase for up to 54 hours. The amount of cytochrome c in the membrane fraction including mitochondria showed a corresponding decrease in a time-dependent manner. The appearance of cytochrome c correlated with the activation of caspase-3-like protease (DEVDase), and a decrease in the amount of the proform of caspase-3 and -6. Furthermore, SB203580, a specific p38 MAPK inhibitor, reduced hypoxia-induced caspase-3 and -6 activation, and resulted in the partial protection of apoptosis in MC3T3E1 osteoblasts.

These findings are of potential clinical relevance. Our *in vitro* results suggest that therapies targeted at the inhibition of caspase-3-like protease activation by hypoxia should abrogate apoptosis in osteoblasts. In addition, antagonism of p38 MAPK may potentially reduce osteoblast damage during physiological hypoxia.

CONCLUSIONS

The aim of this study was to elucidate the possible mechanism of apoptosis in response to hypoxia in MC3T3E1 osteoblasts, the main cells in bone remodeling during orthodontic tooth movement.

MC3T3E1 osteoblasts under hypoxic conditions (2%

oxygen) resulted in apoptosis in a time-dependent manner estimated 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, completely suppressed the DNA ladder in response to hypoxia. 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 are involved in apoptosis, Western blot analysis was performed using anti-caspase-3 or -6 antibody. The 10-kDa protein, corresponding to the active products of caspase-3 and the 10-kDa protein of the active protein of caspase-6 were generated in hypoxia-challenged cells in which processing of the full-length form of caspase-3 and -6 was evident. With a time course similar to this caspase-3 and -6 activation evident, hypoxic stress caused the cleavage of lamin A, which is typical of caspase-6 activity. In addition, the stress elicited the release of cytochrome c into the cytosol during apoptosis. Furthermore, we observed that pre-treatment with SB203580, a selective p38 MAPK inhibitor, attenuated the hypoxia-induced apoptosis. The addition of SB203580 suppressed caspase-3 and -6-like protease activity by hypoxia up to 50%. In contrast, PD98059 had no effect on the hypoxia-induced apoptosis. To confirm the involvement of MAP kinase, JNK/SAPK, ERK, or p38 kinase assay was performed. Although p38 MAPK was activated in response to hypoxic treatment, the other MAPK -JNK/SAPK or ERK- was not or was only modestly activated. These results suggest that p38 MAPK is involved in hypoxia-induced apoptosis in MC3T3E1 osteoblasts.

REFERENCES

1. Kerr JFR, Wyllie AH, Currie AR. Apoptosis : a basic biological phenomenon with wide ranging implications in tissue kinetics. Br J Cancer 1972 : 26 : 239-57.
2. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science 1995 : 267 : 1456-62.
3. Wyllie AH, Kerr JFR, Currie AR. Cell death : the significance of apoptosis. Int Rev Cytol 1980 : 68 : 251-306.
4. Jacobson MD, Raff MC. Programmed cell death and Bcl-2 protection in very low oxygen. Nature 1995 : 374 : 814-6.
5. Shimizu S, Eguchi Y, Kosaka H. et. al. Prevention of hypoxia-induced cell death by Bcl-2 and Bcl-xL. Nature 1995 : 374 : 811-3.
6. Krajewski S, Krajewska M, Ellerby LM. et. al. Release of caspase-9 from mitochondria during neuronal apoptosis and cerebral ischemia. Proc Natl Acad Sci USA 1999 : 96 : 5752-7.
7. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science 1998 : 281 : 1305-8.
8. Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature 1994 : 371 : 346-7.
9. Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell 1998 : 94 : 481-90.
10. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 1997 : 91 : 479-89.
11. Pan G, O'Rourke K, Dixit VM. Caspase-9, Bcl-XL, and Apaf-1 form a ternary complex. J Biol Chem 1998 : 273 : 5841-5.
12. Thornberry NA, Lazebnik Y. Caspases: enemies within. Science 1998 : 281 : 1312-6.
13. Borutaite V, Morkuniene R, Budriunaite A, Krasauskaite D, Ryselis S, Toleikis A, Brown GC. Kinetic analysis of changes in activity of heart mitochondrial oxidative phosphorylation system induced by ischemia. J Mol Cell Cardiol 1996 : 28 : 2195-201.
14. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 1997 : 275 : 1132-6.
15. Pastorino JG, Chen ST, Tafani M, Snyder JW, Farber JL. The overexpression of Bax produces cell death upon induction of the mitochondrial permeability transition. J Biol Chem 1998 : 273 : 7770-5.
16. Yin T, Sandhu G, Wolfgang CD. et. al. Tissue-specific pattern of stress kinase activation in ischemic/reperfused heart and kidney. J Biol Chem 1997 : 272 : 19943-50.
17. Jun CD, Pae HO, Kwak HJ. et. al. Modulation of nitric oxide-induced apoptotic death of HL-60 cells by protein kinase C and protein kinase A through mitogen-activated protein kinases and CPP32-like protease pathways. Cell Immunol 1999 : 194 : 36-46.
18. Boulton TG, Yancopoulos GD, Gregory JS, Slaughter C, Moomaw C, Hsu J, Cobb MH. An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. Science 1990 : 249 : 64-7.
19. Hibi M, Lin A, Smeal T, Minden A, Karin M. Identification of an oncoprotein-and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev 1993 : 7 : 2135-48.
20. Han J, Lee JD, Bibbs L, Ulevitch RJ. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 1994 : 265 : 808-11.
21. Zhang W, Lee JC, Kumar S, Gowen M. ERK pathway mediates

the activation of Cdk2 in IGF-1-induced proliferation of human osteosarcoma MG-63 cells. J Bone Miner Res 1999 : 14 : 528-35.
22. Chae HJ, Chae SW, Weon KH, Kang JS, Kim HR. Signal transduction of thapsigargin-induced apoptosis in osteoblast. Bone 1999 : 25 : 453-8.
23. Nakazawa M, Uehara T, Nomura Y. Koningic acid (a potent glyceraldehyde-3-phosphate dehydrogenase inhibitor)-induced fragmentation and condensation of DNA in NG108-15 cells. J Neurochem 1997 : 68 : 2493-9.
24. Palayoor ST, Macklis RM, Bump EA, Coleman CN. Modulation of radiation-induced apoptosis and G2/M block in murine T-lymphoma cells. Radiat Res 1995 : 141 : 235-43.
25. Conrad PW, Rust RT, Han J, Millhorn DE, Beitner-Johnson D. Selective activation of p38alpha and p38gamma by hypoxia. Role in regulation of cyclin D1 by hypoxia in PC12 cells. J Biol Chem 1999 : 274 : 23570-6.
26. Cheng Y, Deshmukh M, D'Costa A. et. al. Caspase inhibitor affords neuroprotection with delayed administration in a rat model of neonatal hypoxic-ischemic brain injury. J Clin Invest 1998 : 101 : 1992-9.
27. Takahashi A, Alnemri ES, Lazebnik YA. et. al. Cleavage of lamin A by Mch2 alpha but not CPP32: multiple interleukin 1 beta-converting enzyme-related proteases with distinct substrate recognition properties are active in apoptosis. Proc Natl Acad Sci USA 1996 : 93 : 8395-400.
28. Suzuki A, Palmer G, Bonjour JP, Caverzasio J. Regulation of alkaline phosphatase activity by p38 MAP kinase in response to activation of Gi protein-coupled receptors by epinephrine in osteoblast-like cells. Endocrinology 1999 : 140 : 3177-82.
29. Omura T, Yoshiyama M, Shimada T, Shimizu N, Kim S, Iwao H, Takeuchi K, Yoshikawa J. Activation of mitogen-activated protein kinases in in vivo ischemia/reperfused myocardium in rats. J Mol Cell Cardiol 1999 : 31 : 1269-79.
30. Uehara T, Kaneko M, Tanaka S, Okuma Y, Nomura Y. Possible involvement of p38 MAP kinase in HSP70 expression induced by hypoxia in rat primary astrocytes. Brain Res 1999 : 823 : 226-30.
31. Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev 1999 : 79 : 143-80.
32. Su B, Karin M. Mitogen-activated protein kinase cascades and regulation of gene expression. Curr Opin Immunol 1996 : 8 : 402-11.
33. Garrington TP, Johnson GL. Organization and regulation of mitogen-activated protein kinase signaling pathways. Curr Opin Cell Biol 1999 : 11 : 211-8.
34. Rouse J, Cohen P, Trigon S. et. al. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. Cell 1994 : 78 : 1027-37.
35. Raingeaud J, Gupta S, Rogers JS, et.al. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem 1995 : 270 : 7420-6.
36. Kyriakis JM, Avruch J. Sounding the alarm : protein kinase cascades activated by stress and inflammation. J Biol Chem 1996 : 271 : 24313-6.
37. Logan SK, Falasca M, Hu P, Schlessinger J. Phosphatidylinositol 3-kinase mediates epidermal growth factor-induced activation of the c-Jun N-terminal kinase signaling pathway. Mol Cell Biol 1997 : 17 : 5784-90.
38. Xing J, Kornhauser JM, Xia Z, Thiele EA, Greenberg ME. Nerve growth factor activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB serine 133 phosphorylation. Mol Cell Biol 1998 : 18 : 1946-55.
39. Ohta T, Kinoshita T, Naito M. et. al. Requirement of the caspase-3/CPP32 protease cascade for apoptotic death following cytokine deprivation in hematopoietic cells. J Biol Chem 1997 : 272 : 23111-6.
40. Kim CN, Wang X, Huang Y. et. al. Overexpression of Bcl-X(L) inhibits Ara-C-induced mitochondrial loss of cytochrome c and other perturbations that activate the molecular cascade of apoptosis. Cancer Res 1997 : 57 : 3115-20.
41. Zhivotovsky B, Orrenius S, Brustugun OT, Doskeland SO. Injected cytochrome c induces apoptosis. Nature 1998 : 391 : 449-50.
42. Hampton MB, Zhivotovsky B, Slater AF, Burgess DH, Orrenius S. Importance of the redox state of cytochrome c during caspase activation in cytosolic extracts. Biochem J 1998 : 329 : 95-9.

국문초록

저산소 상태로 인한 조골세포 고사기전에서 p-38 MAP kinase의 역할에 관한 연구

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교정력에 의한 치아 이동은 기계적인 힘에 의하여 압박측에는 다양한 구조를 가진 치주 조직에 혈류의 변화가 생기며 국소적으로 산소 장력에도 변화가 생겨 저산소 상태가 유발됨은 이미 확인한 바 있다. 본 연구는 치아 주위 골격을 형성하는 조골세포를 대상으로 교정적 치아 이동과 유사한 시험관내 조건을 설정하여 저산소 상태 시 유발되는 조골세포 고사조절 기전을 규명하고자 시행하였다. 생리적인 저산소증의 실험조건으로 2% 산소상태를 설정하여 저산소 하에서 세포가 고사(apoptosis) 됨을 확인하였고, stress유발 시 많은 관련을 가진 것으로 알려진 p-38 MAPK의 활성을 관찰하였다. 또한 p-38 MAPK의 억제제인 SB203580의 전처치로 인하여 세포의 죽음이 억제됨을 확인하였고, 저산소 상태 시 활성형태로 분절되는 caspase-3, -6및 9등의 세포고사관련 효소들의 활성 형태로의 분절이 억제됨을 확인하였으며 이러한 caspase의 기질인 Lamin-A등의 분절 또한 억제 됨을 밝혔다. 또한 마이토콘드리아 내의 cytochrome c의 세포질내로의 이동 또한 조절됨을 확인함으로써 p-38 MAPK의 조절단계를 시사하여 주고 있다. 본 연구로 치아 이동 시 유발되는 저산소 상태 하에서 발생하는 조골세포의 고사 조절에 p-38 MAPK가 관여함을 확인하였다.

주요 단어 : 저산소증, 세포 고사, 조골세포, p-38 MAPK