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PDTC Inhibits TNF-α-Induced Apoptosis in MC3T3E1 Cells

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Osteoblasts are affected by TNF-α overproduction by immune cells during inflammation. It has been suggested that functional NF-κB sites are involved in TNF-α-induced bone resorption. Thus, we explored the effect of pyrrolidine dithiocarbamate (PDTC), which potently blocks the activation of nuclear factor (NF-κB), on the induction of TNF-α-induced activation of JNK/SAPK, AP-1, cytochrome c, caspase and apoptosis in MC3T3E1 osteoblasts. Pretreatment of the cells with PDTC blocked TNF-α-induced NF-κB activation. TNF-α-induced activation of AP-1, another nuclear transcription factor, was suppressed by PDTC. The activation of c-Jun N-terminal kinase, implicated in the regulation of AP-1, was also down regulated by PDTC. TNF-α-induced apoptosis, release of cytochrome c and subsequent activation of caspase-3 were abolished by PDTC. TNF-α-induced apoptosis was partially blocked by Ac-DEVD-CHO, a caspase-3 inhibitor, suggesting that caspase-3 is involved in TNF-α-mediated signaling through NF-κB in MC3T3E1 osteoblasts. Thus, these results demonstrate that PDTC, has an inhibitory effect on TNF-α-mediated activation of JNK/SAPK, AP-1, cytochrome c release and subsequent caspase-3, leading to the inhibition of apoptosis. Our study may contribute to the treatment of TNF-α-associated immune and inflammatory diseases such as rheumatoid arthritis and periodontal diseases.

Key Words: TNF-α, Pyrrolidine dithiocarbamate, NF-κB, Osteoblasts, Apoptosis, c-Jun N-terminal kinase

INTRODUCTION

The transcription factor NF-κB is a critical regulator of cytokine-inducible gene expression (Baeuerle, 1994). Pro-inflammatory cytokines, such as TNF-α and interleukin-1, allow rapid nuclear translocation of NF-κB through degradation of IκB inhibitory cytoplasmic retention proteins (Baeuerle, 1994; Verma et al, 1995). Genes regulated by nuclear NF-κB include those involved in inflammatory response such as hematopoietic growth factors, chemokines, and leukocyte adhesion molecules (Baeuerle, 1994). A variety of inflammatory stimuli, including TNF, IL-1, and LPS, activate NF-κB (Manna et al, 1998). Most of these stimuli also activate the nuclear transcription factor, activation protein 1 (AP-1). AP-1 consists of a homodimer and heterodimers of the Jun and Fos families (c-Fos, FosB, Fra 1, and Fra2) and is regulated in part by c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (Liu et al, 1996; Xia et al, 1995). Most of the stimuli that activate NF-κB and AP-1 also induce apoptosis. Although many reports suggest that NF-κB plays an important role in osteoblasts, the mechanism of action by which the transcription factor exerts its effects remains a matter of debate.

Cytochrome c redistribution from mitochondria to cytosol occurs in intact cells during apoptosis (Bossy-Wetzel et al, 1998). Cytochrome c is a 12-kDa protein which functions in the mitochondrial electron transport chain. At physiological ionic strength, cytochrome c diffuses in the aqueous phase between the inner and outer membranes (outer compartment) between complex III (cytochrome bc_1) and complex IV (cytochrome aa_3) (Cortese et al, 1993; Gupte et al, 1988). In response to certain apoptotic stimuli, cytochrome c is released from mitochondria (Liu et al, 1996; Kluck et al, 1997; Yang et al, 1997). The released cytochrome c induces the formation of a caspase activating complex.

On the basis of observations that their inhibitors or negative mutants inhibit apoptosis induced by various stimuli (Yuan et al, 1993; Tewari et al, 1995; Chae et al, 1999). The family of cysteine proteases is implicated in apoptotic cell death. In fact, we have recently observed that caspase-3 alone is related with various stimuli such as thapsigargin in MC3T3E1 cells (Brennan & Oneill, 1995). Among these caspases, caspase-3 appears to be an attractive candidate as a putative mediator of apoptosis.

In the present study, we investigated the effect of pyrrolidine dithiocarbamate (PDTC), a potent NF-κB inhibitor, on TNF-α-induced apoptosis in MC3T3E1 osteoblasts. Since NF-κB activation has been proposed to play a role in TNF-α-induced cell death (Hibi et al, 1993). We also investigated the effect of PDTC on TNF-α-activated JNK/SAPK, AP-1, cytochrome c, caspase, and apoptosis.

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ABBREVIATIONS: PDTC, pyrrolidine dithiocarbamate; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-kappa B; AP-1, activation protein-1.

METHODS

Reagents

Recombinant mouse TNF-α (γTNF-α, 5 units/ ng) was obtained from Genzyme Co (Cambridge, MA, USA), and the pyrrolidine dithiocarbamate (PDTC) was from Sigma Chemical Co. (St. Louis, MO). All culture wares were purchased from Nunc Inc. (North Aurora Road, IL, USA), and other culture reagents, including α-Minimum Essential Medium (α-MEM), Hank's balanced salt solution, and fetal bovine serum (FBS) were from GIBCO Co. (Gaithersburg, MD, USA).

Cell culture

A murine osteoblastic cell line, MC3T3E1 cells, was maintained in α-MEM supplemented with 10% FBS, penicillin G (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM).

DNA extraction and electrophoresis

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 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% agarose electrophoresis at 100 V for 1 hr. DNA was visualized by staining with ethidium bromide under UV light.

In vitro immunocomplex kinase assay for JNK1

Phosphotransferase activity of JNK1 was measured by modification of the procedure previously described (Kluck et al, 1997). Briefly, MC3T3E1 cells (2×10^6 cells/each group) were treated with 20 ng/ml TNF-α for various time periods and lysed in a lysis buffer (EB buffer: 1% Triton X-100, 10 mM Tris, pH 7.6, 50 mM NaCl, 1 mg/ml aprotinin, 5 mM EDTA, 50 mM NaF, 0.1% 2-mercaptoethanol, and 100 μM sodium orthovanadate). The protein concentration in the supernatant was determined and then incubated with anti-JNK1 antibodies (Santa Cruz Inc. Santa Cruz, CA). After incubation on ice for 3 hr, 100 μl of 10% suspension of formalin- fixed *Staphylococcus aureus* (Calbiochem, La Jolla, CA) was added to anti-JNK1 immunoprecipitates, and further incubated on ice for 1 hr. The absorbed immune complex was washed twice with EB buffer and PAN buffer (10 mM PIPES buffer, pH 7.0, 1% aprotinin, 100 mM NaCl). The immunocomplex was mixed with 2 μg of GST-c-Jun $NT_{1\text{-}79}$ proteins as a substrate in 30 μl of the reaction buffer, containing 2 μM cold ATP, 2 mM DTT, 20 mM $MgCl_2$, 2 μCi [γ^{32}-P] ATP, and 20 mM Tris-HCl, pH 7.5 and the mixture was incubated at 30°C for 20 min. The reaction was terminated by adding 3×SDS-PAGE sample buffer, and the products were separated on 12% SDS-PAGE, and the phosphorylated c-jun was visualized by autoradiography. A PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A) was used to quantify band intensity.

Electrophoretic mobility shift assay (EMSA)

The nucleus from TNF-α-treated MC3T3E1 cells was extracted by modification of the procedure described by Dignam et al (Kim et al, 1998). Briefly, binding reaction mixture (20 μl) contained 2 μg poly (dI-dC) (Pharmacia Fine Chemicals, Piscataway, NJ), 50,000~100,000 cpm (Cerenkov) of ^{32}P-labeled DNA probe (>3,000 Ci/mmol), 1 μl 1% NP-40 containing buffer D, 20 μg BSA, and binding buffer. Reactions were started by the addition of cell extracts and allowed for 30 min before electrophoresis. The reaction products were analyzed on native 4% polyacrylamide gels. Dried gels were visualized by autoradiography. A PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A) was used to quantify band intensity.

Measurement of caspase-1 and caspase-3-like protease activity

To measure the caspase protease activity, MC3T3E1 cells were lysed in a lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 1 mM PMSF, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 2 mM dithiothreitol, 10 mM Tris/HCl, pH 8.0) on ice for 30 min and cleared at 15,000×g for 15 min. The activity of caspase-1 and -3-like proteases was measured by cleavage of 100 μM 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, 1 mM PMSF, 1 mg/ml aprotinin, 1 mg/ml leupeptin, and 2 mM dithiothreitol. The amounts of released 7-amino-4-methylcoumarin (AMC) were measured spectrofluorometrically (Molecular Devices, Palo Alto, CA, USA) with excitation at 380 nm and emission at 460 nm.

Cytochrome c measurements

Mitochondrial fractions were prepared from 1×10^7 MC3T3E1 cells by differential centrifugation in buffer containing 250 mM sucrose as described previously(Yang et al 1997). Protein samples (25 μg) were loaded on 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 antibody (Pharmingen, San Diego, Calcif) and secondary anti-mouse horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology), and then developed with enhanced chemiluminescence (Amersham Life Science).

Quantitation of DNA fragmentation

DNA fragmentation was assayed essentially as reported (Li et al, 1997). Briefly, following cell lysis, intact chromatin (pellet) was separated from DNA fragments (supernatant) by centrifugation. DNA contents were quantitated using the diphenylamine reagents (Baeuerle & Baichwal 1997). The percentage of fragmented DNA was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet.

Statistic analysis

Statistical analysis was performed using the Student's t-test, and a p value less than 0.05 was selected as the level of significance.

RESULTS

TNF-α induced the apoptotic death of MC3T3E1 cells

When treated with 20 ng/ml TNF-α, MC3T3E1 cells underwent apoptotic cell death. As shown in Fig. 1A, treatment with TNF-α (20 and 40 ng/ml) induced DNA fragmentation in MC3T3E1 cells. Oligosomal DNA fragmentation, consistent with the onset of apoptosis, was evident after 24 hr in TNF-α-treated cells. Using PDTC, a potent inhibitor of NF-κB and antioxidant, we analyzed DNA from these cells by agarose gel electrophoresis for evidence of fragmentation (Fig. 1B). When pretreated with PDTC (50 μM) for 30 min, DNA fragmentation was substantially inhibited in TNF-α-treated cells. However, pretreatment of other antioxidants such as N-acetyl-L-cysteine (1~10 mM), superoxide dismutase (20~400 IU) or catalase (20~2000 IU) did not suppress subsequent apoptosis induced by TNF-α (data not shown). Next, we examined apoptotic chromatin changes in PDTC untreated or -treated MC3T3E1 osteoblasts by fluorescence microscopy. PDTC at 50 μM inhibited TNF-α-induced apoptosis in MC3T3E1 cells (Fig. 1C). These results show that NF-κB is an important signal of TNF-α-induced apoptosis in MC3T3E1 cells.

PDTC inhibits TNF-α-induced NF-κB binding in MC3T3E1 cells

We next examined using the gel mobility shift assay, whether the presence of NF-κB could be detected in the TNF-α treated MC3T3E1 osteoblasts, and if so, also whether NF-κB binding activity in the MC3T3E1 cells was inhibited by PDTC. As shown in Fig. 2A, the binding of nuclear extracts from TNF-α-treated MC3T3E1 cells to the consensus sequence of the binding site for NF-κB was observed, however, NF-κB binding clearly disappeared by treatment with PDTC at 50 μM (Fig. 2B). The finding is consistent with the report that PDTC reversibly suppressed the release of the inhibitory IκB subunit from the latent cytoplasmic NF-κB form in cells treated with phorbol ester, interleukin 1, and tumor necrosis factor α (Chen et al, 1996).

PDTC inhibits TNF-α-induced JNK/SAPK activity in MC3T3E1 cells

It has earlier been shown that the JNK pathway is involved in radiation-induced apoptosis (Verheij et al, 1996), and that the JNK cascade is required for apoptosis

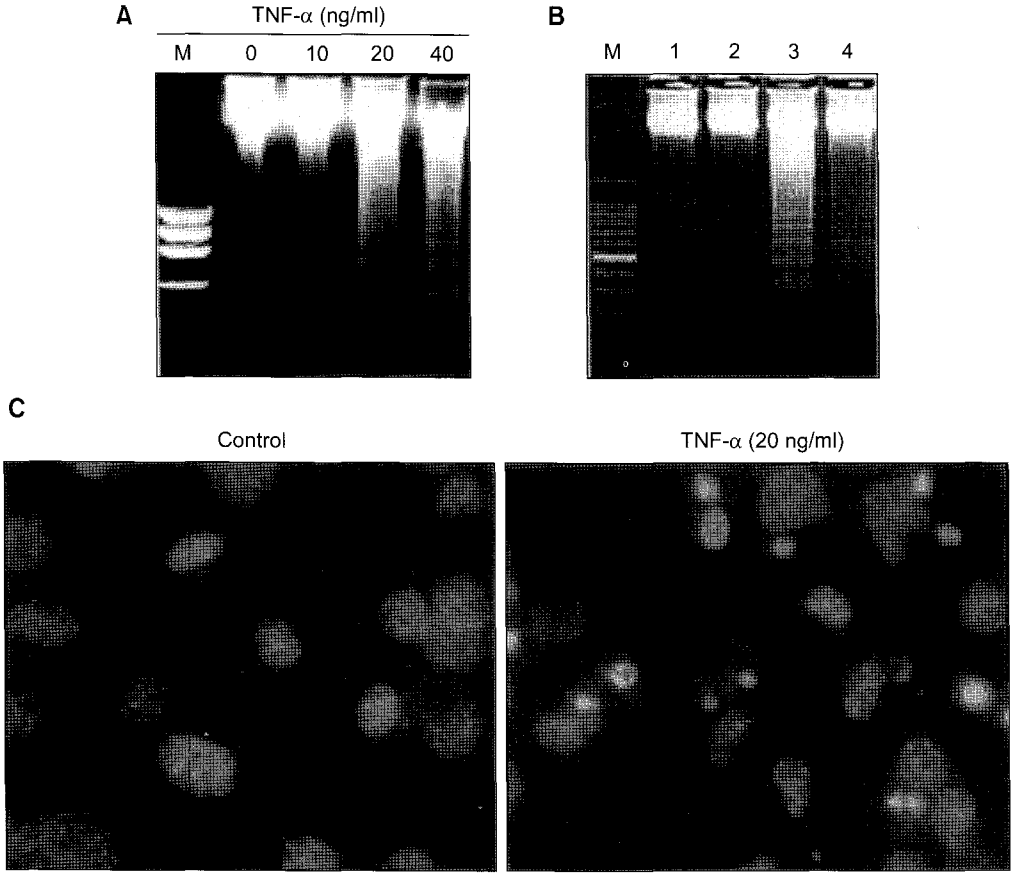

Fig. 1. Inhibition of TNF-α-induced apoptosis by pyrrolidine dithiocarbamate (PDTC) in MC3T3E1 osteoblasts (A). MC3T3E1 cells were treated with TNF-α (10, 20, or 40 ng/ml) for 24 h (B). MC3T3E1 cells pretreated with PDTC (50 μM) were treated with TNF-α (20 ng/ml) for 24 h. The cells were then harvested and assayed for DNA fragmentation (C). MC3T3E1 cells were treated with TNF-α (20 ng/ml) in the presence or absence of PDTC (50 μM) for 24 hr. The fixed cells were incubated with Hoechst 33258 (2.5 μg/ml in PBS), and nuclear staining was examined with a fluorescence microscope.

induced by growth factor withdrawal (Xia et al, 1995) or ceramide treatment (Zhao et al, 1999). As shown in Fig. 3A, TNF-α increased phosphotransferase activity of JNK1 toward c-Jun protein at 0.5 and 1 hr, and this transient increase of JNK1 activity returned to the basal level at around 2 hr after the treatment of TNF-α. We then tested if the treatment of PDTC had a suppressive effect on TNF-α-induced JNK activation in MC3T3E1 osteoblasts. As shown in Fig. 3B, PDTC (50 μM) reduced TNF-α-stimulated JNK activation to the basal level.

PDTC inhibits TNF-α-induced AP-1 binding in MC3T3E1 cells

AP-1 is known to be composed of either heterodimer of Jun family proteins with Fos family proteins or homodimer of Jun family proteins (Xia et al, 1995; Liu et al, 1996). Thus, we investigated whether TNF-α might activate AP-1, since it transiently activated the upstream molecule of c-Jun, JNK1, in a time-dependent manner. As shown in Fig. 4A, the nuclear extract (5 μg) of MC3T3E1 cells was used to carry out EMSA for AP-1, and TNF-α clearly increased the binding activity of nuclear extract to oligonucleotide

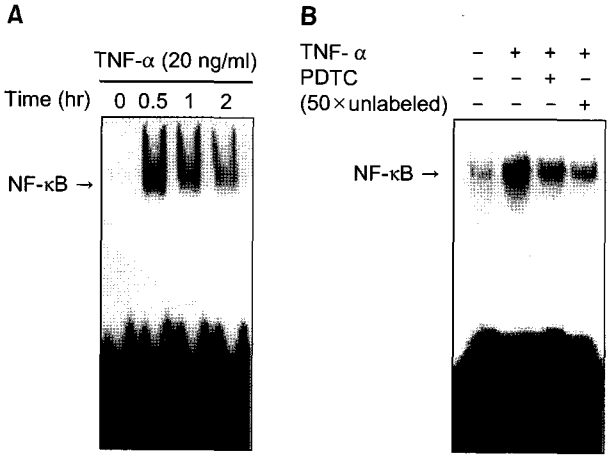

Fig. 2. Inhibition of TNF-α-induced NF-κB binding by PDTC in MC3T3E1 osteoblasts (A). MC3T3E1 cells were treated with TNF-α (20 ng/ml) for the indicated time points (B). MC3T3E1 cells were treated with TNF-α (20 ng/ml) in the presence or absence of PDTC (50 μM) for 0.5 hr, and then nuclear extracts were prepared and assayed for NF-κB bindings as described under Methods. Addition of a 50-fold excess of unlabeled consensus NF-κB oligomer completely inhibited the binding.

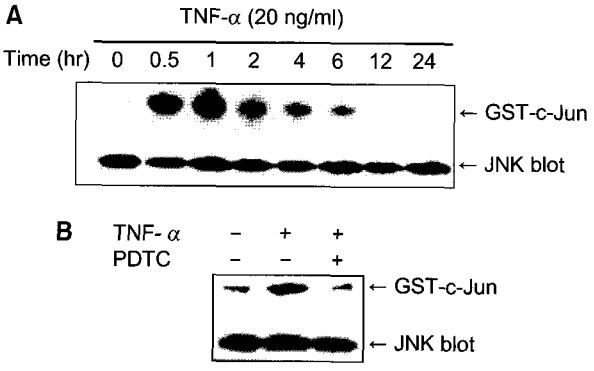

Fig. 3. Inhibition of TNF-α-induced JNK/SAPK activation by PDTC in MC3T3E1 osteoblasts (A). MC3T3E1 cells were treated with TNF-α (20 ng/ml) at different time points as indicated (B). MC3T3E1 cells were treated with TNF-α (20 ng/ml) in the presence or absence of PDTC (50 μM) for 1 hr. Endogenous JNK activity was examined by immunocomplex assays.

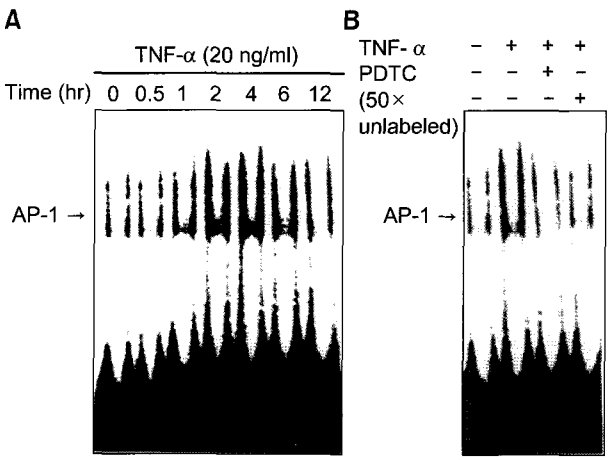

Fig. 4. Inhibition of TNF-α-induced AP-1 activity by PDTC in MC3T3E1 osteoblasts (A). MC3T3E1 cells were treated with TNF-α (20 ng/ml) at different time points as indicated. (B). MC3T3E1 cells were treated with TNF-α (20 ng/ml) in the presence or absence of PDTC (50 μM) for 2 hr, and then nuclear extracts were prepared and assayed for AP-1 binding as described under Methods. Addition of a 50-fold excess of unlabeled consensus AP-1 oligomer completely inhibited the binding.

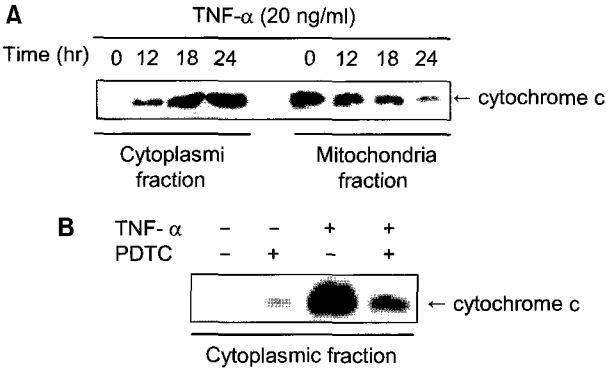

Fig. 5. Inhibition of TNF-α-induced cytochrome c release by PDTC in MC3T3E1 osteoblasts (A). MC3T3E1 cells were treated with TNF-α (20 ng/ml) at different time points as indicated (B). MC3T3E1 cells were treated with TNF-α (20 ng/ml) in the presence or absence of PDTC (50 μM) for 18 hr, and then mitochondrial and cytoplasmic fractions were prepared, separated on SDS-PAGE and transferred onto a nitrocellulose membrane. Cytochrome c was visualized by Western blot analysis.

probe of AP-1 at 2 hr. Fig. 4B shows that PDTC reduced the activation of AP-1 to the basal level, suggesting that PDTC blocked TNF-α-stimulated phosphotransferase activity of JNK1 and subsequent activation of AP-1 transcription in MC3T3E1 osteoblasts.

PDTC inhibits TNF-α-induced cytochrome c release in MC3T3E1 cells

Cytochrome c is released into cytoplasm to induce apoptosis in carrot and the stereotypical DNA ladder can be observed (Li et al, 1997). Therefore, cytochrome c can be an apoptosis inducer (Abbadie et al, 1993). The steps involved in TNF-α signaling through cytochrome c in osteoblasts have not yet been investigated. Therefore, the amounts of cytochrome c in the mitochondria and cytosol fractions were measured by Western blot analysis. As shown in Fig. 5A, induction of apoptosis was associated with cytochrome c release into the cytosol as determined by immunoblotting. Next, we observed that PDTC had inhibitory effect on TNF-α-induced cytochrome c release in MC3T3E1 osteoblasts (Fig. 5B). Thus, the inhibition of apoptosis by PDTC is associated with reduction of cytochrome c release.

PDTC inhibits TNF-α-induced caspase-3-like protease activity in MC3T3E1 cells

Recently, activation of caspase family proteases has been shown to be critically required to initiate the induction of apoptosis (Miura et al, 1993; Kluck et al, 1997). Our results showed that TNF-α-induced the activation of caspase-3-like protease in MC3T3E1 osteoblasts at 12 hr, reached a maximum at 18 hr, and returned to the basal level (Fig. 6A). However, the activity of caspase-1-like proteases was not changed by the addition of TNF-α. We also observed that the inhibition of apoptosis by PDTC was associated with suppressed caspase-3-like activity. As shown in Fig. 6B, pretreatment of the cells with PDTC efficiently blocked subsequent TNF-α-induced caspase-3-like protease activation. In addition, TNF-α-induced rapid loss of viability associated with DNA fragmentation was partially inhibited by Ac-DEVD-CHO (200 μM), a caspase-3-like protease inhibitor (Fig. 6C). As expected, Ac -YVAD-CHO (200 μM), a caspase-1-like protease inhibitor, had no inhibitory effect on TNF-α-induced apoptosis. Thus, our data show that caspase-3 alone is required to induce apoptosis in MC3T3EI cells, indicating that PDTC has a regulatory function in TNF-α-induced apoptosis via inhibition of caspase-3 in MC3T3E1 cells.

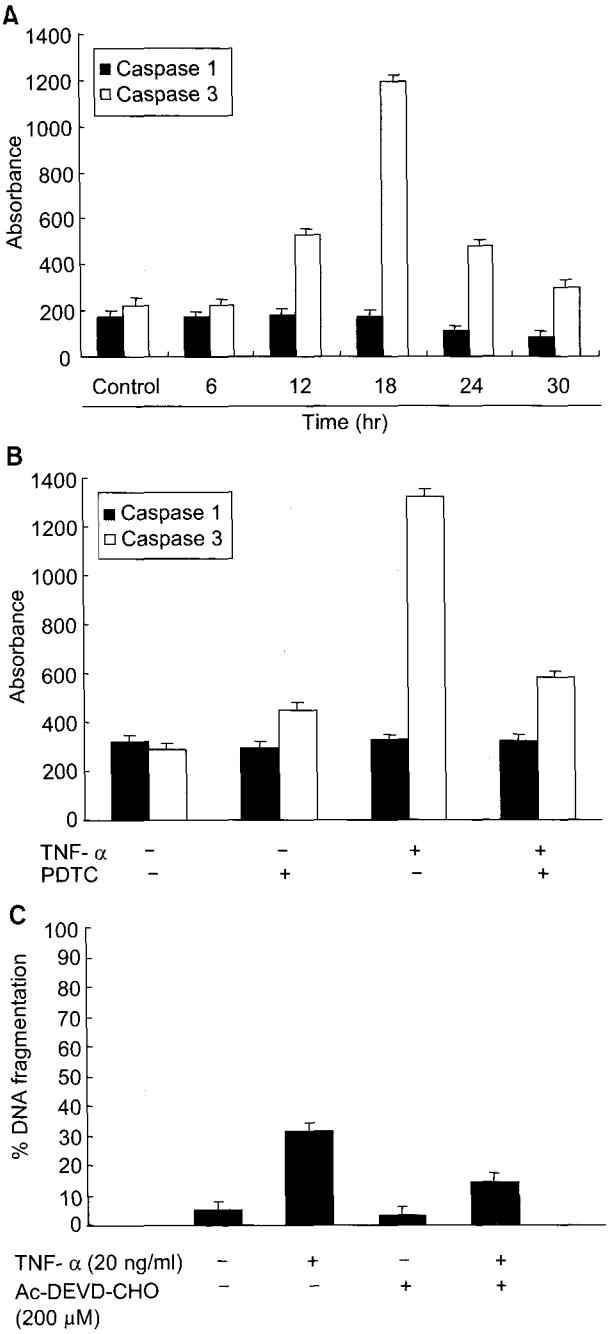

Fig. 6. Inhibition of TNF-α-induced caspase-3 activation by PDTC in MC3T3E1 osteoblasts (A). MC3T3E1 cells were treated with TNF-α (20 ng/ml) at different time points as indicated (B). MC3T3E1 cells were treated with TNF-α (20 ng/ml) in the presence or absence of PDTC (50 μM) for 18 hr, and then caspase activity was measured as described in Methods (C). MC3T3E1 cells were treated with TNF-α (20 ng/ml) in the presence or absence of Ac-DEVD-CHO (200 μM) for 24 hr, and cells were then harvested and assayed for DNA fragmentation assay as described in Methods.

DISCUSSION

In this study, we report that TNF-α-induced apoptosis is significantly inhibited by PDTC, a potent NF-κB inhibitor. Although high dose of PDTC had cytotoxic effect on MC3T3E1 cells, pretreatment of the cells with PDTC was able to block TNF-α-induced apoptosis (Fig. 1A). Our findings, therefore, indicate that PDTC promotes survival by inhibiting TNF-α-induced NF-κB activation. Although there is no direct evidence to indicate that activation of NF-κB by TNF-α causes the apoptosis of MC3T3E1 cells, many studies indicate that the regulation of NF-κB is one of the most important apoptotic signaling pathways. For example, Abbadie et al (Bessho et al, 1994) reported that the overexpression of c-Rel induced apoptosis of avian bone marrow cells, and Bessho et al (Baldwin, 1996) also showed that treatment of human leukemia cells and thymocytes with a protease inhibitor, pyrrolidine dithiocarbamate

(PDTC), which inhibits activation of NF-κB, prevented their apoptosis. In addition, radiation or agents such as lipopolysaccharides and TNF-α triggered the hydrolysis of membrane phospholipids to produce ceramide, which in turn activated NF-κB and induced apoptosis (Beg et al, 1996). In the contrary, however, Beg et al (Wang et al, 1996) showed that a considerable apoptosis occurred in hepatocytes in Rel A (p65)-deficient embryos, and Wang et al (Van Antwerp et al, 1996) and Antwerp et al (Welsh et al, 1996) independently demonstrated the anti-apoptotic role of NF-κB, using cell lines transfected with the dominant negative IκBα. It is not known at present whether NF-κB is involved in promotion of apoptosis in certain cells and if also responsible for inhibition of apoptosis in other cells. Neverthless, in agreement with the former contention, this study indicates that the signaling cascade mediated by NF-κB may be important in the death of osteoblasts.

We have found that TNF-α-induced JNK/SAPK activation is regulated by PDTC (Fig. 3B). The c-Jun N-terminal kinase (JNK1) is activated by cellular stress agonists such as proinflammatory cytokines and chemical stress(Raines et al, 1993; Minden et al, 1994). In addition, PDTC blocked TNF-α-induced AP-1 activation (Fig. 4B). AP-1 activation has been shown to be regulated by JNK through phosphorylation of c-Jun (Chen et al, 1998). Since we found that PDTC also blocked JNK activation by TNF-α (Fig. 3B), it is possible that PDTC suppresses AP-1 transcriptional activation through JNK. Although there is an earlier reports that JNK activation is needed for apoptosis induced by certain stimuli (Kim et al, 1998), whether JNK activation is needed for TNF-α-induced apoptosis is not known. Neverthless, it is possible that PDTC exerts its effects on TNF-α-induced apoptosis through inhibition of JNK.

As noted earlier, PDTC inhibited TNF-α-induced apoptosis. The pathway leading to cytotoxicity/apoptosis is known to involve the release of cytochrome c by mitochondria and the downstream caspase activation (Zhao et al, 1999). These data presented are consistent with the hypothesis of cytochrome c release at or before the onset of the nuclear changes of apoptosis. In our experiments, caspase-3 processing and the release of cytochrome c preceded TNF-α-induced apoptosis, thus indicating a temporal and perhaps causal relationship. Furthermore, the release of cytochrome c into the cytoplasm was blocked by PDTC, suggesting that NF-κB propagate a signal that determines mitochondrial cytochrome c release. As shown in Fig. 6A, caspase-1 cannot be processed by TNF-α in MC3T3E1 cells. Thus, an YVAD-inhibitable caspase-1-like protease is probably not the target for TNF-α. Our study showed that, Ac-DEVD-CHO, a caspase-3 inhibitor, partially blocked TNF-induced apoptosis in MC3T3E1 cells (Fig. 6C), suggesting that a DEVD-CHO-inhibitable caspase controls TNF-α apoptosis and resides upstream of mitochondria because of its ability to block cytochrome c release.

Our data suggest that PDTC-induced cell survival may involve not only the inhibition of cytochrome c release following that of caspase-3 stimulation, but also a JNK/SAPK-dependent pathway leading to the phosphorylation of c-jun and subsequent AP-1 activaton via the inhibition of NF-κB activation in TNF-α-treated MC3T3E1 cells.

Consequently, the inhibition of the NF-κB pathway by PDTC may constitute a single and critical target for therapeutic intervention in those pathologies associated with abnormal cytokine secretion and TNF-α-induced apoptosis in osteoblasts.

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