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TGF-β1 inhibits multiple caspases induced by TNF-α in murine osteoblastic MC3T3-E1 cells

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Abstract

Tumor necrosis factor α (TNF-α) is a proinflammatory cytokine that induces apoptosis in a number of cell systems, including osteoblasts. Transforming growth factor β1 (TGF-β1) is an abundant growth factor that is known to stimulate bone formation. This study was designed to examine the role of TGF-β1 on TNF-α-induced apoptosis in murine osteoblastic MC3T3-E1 cells. Total RNA was extracted from MC3T3-E1 cells treated with 20 ng/ml of TNF-α, 10 ng/ml of TGF-β1, or combination, for 6 h. TNF-α exerted a variety of effects on the apoptotic gene expression in osteoblasts. Ribonuclease protection assays (RPA) revealed that TNF-α upregulated the mRNA levels of caspase-1, -7, -11, -12, and FAS. Western blot analysis showed enhanced processing of caspase-1, -7, -11, and -12, with the appearance of their activated enzymes 24 h after TNF-α treatment. In addition, caspase-3-like activity was significantly activated following TNF-α treatment. Levels of cleaved poly(ADP–ribose) polymerase and FAS protein were also elevated by TNF-α. Finally, Hoechst staining, terminal deoxynucleotidyl-transferase nick-end labeling (TUNEL) assay, and oligonucleosome ELISA all indicated that TNF-α induced apoptosis. In contrast, the addition of TGF-β1 attenuated all of the aforementioned effects of TNF-α. Our results demonstrate that TGF-β1 can decrease TNF-α-induced apoptosis in murine osteoblasts at least in part by attenuating TNF-α-induced caspase gene expression.
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Keywords: TGF-β1; TNF-α; MC3T3-E1 cells; Caspases; Apoptosis

1. Introduction

Apoptosis is crucial for a number of processes including development, tissue homeostasis, viral infection, and cancer [1–3]. It is characterized by cell shrinkage, membrane blebbing, chromatin condensation, and nuclear DNA degradation. Caspases, cysteine proteases with aspartate specificity, are important mediators of apoptosis. The activation of caspases arises from a number of mechanisms including transcriptional activation, regulation of IAP proteins, auto-activation, or cleavage by other caspases [4–6]. To date, 14 members of the caspase family have been isolated. They can be categorized into three groups [7]: initiator caspases (-8, -9, and -10), effector caspases (-3, -6, and -7), and inflammation caspases (-1 and -11).

Bone turnover is controlled by two main types of cells, osteoblasts and osteoclasts, which are responsible for bone formation and resorption, respectively [8,9]. Accumulating evidence indicates that apoptosis plays a central role in the regulation of bone turnover [10,11]. In fact, Jilka et al. [12] demonstrated that apoptosis is the main fate for most osteoblasts. Because bone diseases such as osteoporosis can be caused by a relatively decreased activity or reduced numbers of osteoblasts, prevention of osteoblast apoptosis can be an extremely important clinical goal.

Numerous cytokines and growth factors are present in the bone environment; some are pro-apoptotic, while others are anti-apoptotic [13]. Tumor necrosis factor α (TNF-α), a proinflammatory cytokine secreted by monocytes, plays a key role in abnormal bone remodeling and inflammatory bone diseases [14]. Importantly, it is a potent inducer of apoptosis in osteoblasts [15,16]. TNF-α induces apoptosis by interacting with its receptors (TNF-R1 and TNF-R2) and death receptors such as FAS [17,18]. The roles of specific caspases and TNF-receptor-related proteins in TNF-α-induced apoptosis in osteoblasts are not clearly understood.

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Transforming growth factor β1 (TGF-β1) is an abundant growth factor produced by a number of cells including osteoblasts [19]. It stimulates the synthesis of extracellular matrix components such as fibronectin, collagen, osteonectin, and integrins [20,21]. Administration of TGF-β1 has been reported to enhance cancellous bone formation in juvenile and adult rat bone and fracture healing [22,23]. TGF-β1 has been shown to regulate apoptosis in osteoblasts [12]. However, the molecular basis for the protective effect has not been determined in any detail.

In this report, we studied the involvement of caspases and TNF-receptor proteins in TNF-α-induced apoptosis and examined if TGF-β1 exerts its effect by counteracting TNF-α in murine osteoblastic MC3T3 cells. We found that TNF-α induced apoptosis in murine osteoblastic MC3T3-E1 cells by upregulating FAS expression and by activating caspase-1, -3, -7, -11, and -12. Furthermore, the addition of TGF-β1 attenuated the effects of TNF-α. Our results indicate that TGF-β1 is a potent inhibitor of TNF-α-induced apoptosis in osteoblasts.

2. Materials and methods

2.1. Cell culture and materials

Murine osteoblastic MC3T3-E1 cells were cultured in α-modified MEM (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 25 μg/ml gentamicin at 37 °C in a humidified atmosphere of 5% CO_2. Cells were serially subcultured by treatment with trypsin and were used for experimentation between passages 5 and 15. Human recombinant TNF-α and TGF-β1 were purchased from R&D Systems (Minneapolis, MN). α-[^{32}P]UTP was obtained from ICN (Costa Mesa, CA). Fetal bovine serum was obtained from Biofluid Division-Biosource International (Rockville, MD). Mouse apoptosis gene templates mAPO1, mAPO2, and mAPO3 were obtained from BD Pharmingen (San Diego, CA). Caspase-1 antibody was purchased from Upstate Biotechnology (Lake Placid, NY). FAS antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Caspase-7, cleaved caspase-9, and cleaved PARP antibodies were obtained from Cell Signaling Technology (Beverly, MA). Rat monoclonal caspase-11 and -12 antibodies were generous gifts from Dr. Junying Yuan of Harvard Medical School. Mouse monoclonal actin antibodies (clone AC-40) were purchased from Sigma. Caspase-3 cellular activity assay kit was obtained from Biomol (Plymouth Meeting, PA).

2.2. RPA

MC3T3-E1 cells were seeded into 100-mm dishes containing α-MEM supplemented with 10% FBS. Near confluent cells were changed to α-MEM containing 0.5% FBS for 16 h before the addition of 20 ng/ml of TNF-α, 10 ng/ml of TGF-β1, or both, for 6 h. Total RNA was extracted using acid guanidinium thiocyanate–phenol–$CHCl_3$ extraction method. RPA was performed according to manufacturer's instructions (BD Pharmingen). Briefly, murine apoptosis template sets mAPO-1 (caspases 1, 2, 3, 6, 7, 8, 11, 12, and 14), mAPO-2 (bcl-w, bfl1, bcl-x, bak, bax, bcl-2, bad), and mAPO-3 (caspase 8, FAS L, FAS, FADD, FAP, FAF, TRAIL, TNFRp55, TRADD, RIP) were labeled with [α-^{32}P]UTP. RNA (10 μg) and labeled probes were hybridized and the protected mRNAs were resolved on a 5% denaturing polyacrylamide gel. Intensity of the band was scanned by a Digital Imaging System (Alpha Innotech, San Leandro, CA).

2.3. Western blot analysis

Near confluent MC3T3-E1 cells in 100-mm dishes were incubated with α-MEM containing 0.5% FBS for 16 h before treatment with TNF-α, TGF-β1, or both, for 24 h. Cells were lysed in 2× SDS gel sample buffer and sonicated. Protein concentration was determined by Coomassie dye binding assay (BioRad, Hercules, CA). Aliquots of 50 μg of lysates were electrophoresed on 12% SDS-PAGE and transferred to nitrocellulose membranes. Western blot analysis was carried out with antibodies against caspase-1, -7, -11, -12, cleaved PARP, FAS, and actin. Appropriate secondary antibody conjugated to horseradish peroxidase was then added for 1.5 h. Antigen–antibody complex was detected using enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Piscataway, NJ). Intensity of the band was subjected to image analysis.

2.4. Caspase-3-like activity assay

Near confluent MC3T3-E1 cells in 100-mm dishes were incubated with α-MEM containing 0.5% FBS for 16 h before treatment with TNF-α, TGF-β1, or both, for 24 h. Cells were lysed with a buffer containing 50 mM Hepes, pH 7.4, 0.1% CHAPS, 0.1% NP-40, 5 mM DTT, 0.1 mM EDTA at 4 °C for 20 min. Protein concentration was determined by Coomassie dye binding assay. Cell homogenate containing 40 μg of proteins was assayed for caspase-3-like activity in the presence of 200 μM colorimetric substrate Ac-DEVD-pNA at 37 °C for 4 h according to manufacturer's protocol. An aliquot of cell lysate was treated with 0.1 μM of inhibitor Ac-DEVD-CHO to measure the nonspecific hydrolysis of Ac-DEVD-pNA. Human caspase-3 was included as a positive control. Results were expressed as absorbance at 405 nm/mg protein.

2.5. Hoechst stain

Near confluent MC3T3-E1 cells on 12-mm coverslips (precoated with 0.01% poly L-lysine) were incubated with α-MEM containing 0.5% FBS for 16 h before treatment with TNF-α, TGF-β1, or both, for 24 h. Cells were fixed

with 4% paraformaldehyde for 30 min and stained with 10 μg/ml of Hoechst 33258 dye at room temperature for 10 min. After extensive washing, cells were examined under a microscope equipped for epifluorescence illumination.

2.6. Terminal deoxynucleotidyl-transferase nick-end labeling (TUNEL) assays

TUNEL assay was performed with an in situ apoptosis detection kit (Trevigen, Gaithersburg, MD) according to manufacturer's instructions with minor modifications. Briefly, cells on 12-mm coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in phosphate buffered saline on ice for 5 min before TUNEL staining. In this procedure, nuclei undergoing apoptosis were stained with TACS™ Blue Label chromogenic substrate, and Nuclear Fast Red was used as a counterstain. Positive and negative controls were carried

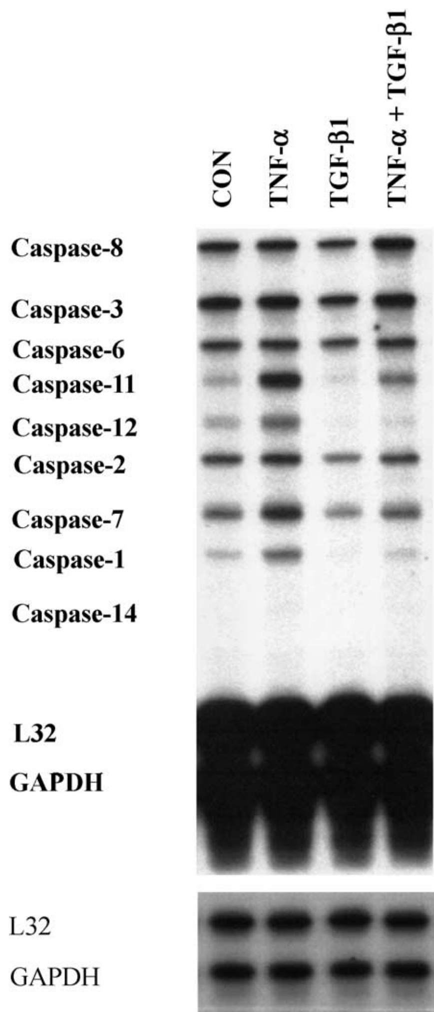

Fig. 1. Caspase mRNA expression in MC3T3-E1 cells. RNA was extracted from MC3T3-E1 cells treated with TNF-α, TGF-β1, or both, for 6 h. RPA was performed with mAPO1 template set as described in the Materials and methods. The specific mRNA transcripts are labeled using the nomenclature from the supplier. Lower panel shows shorter exposure of L32 and GAPDH bands. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. The results are representative of six separate experiments.

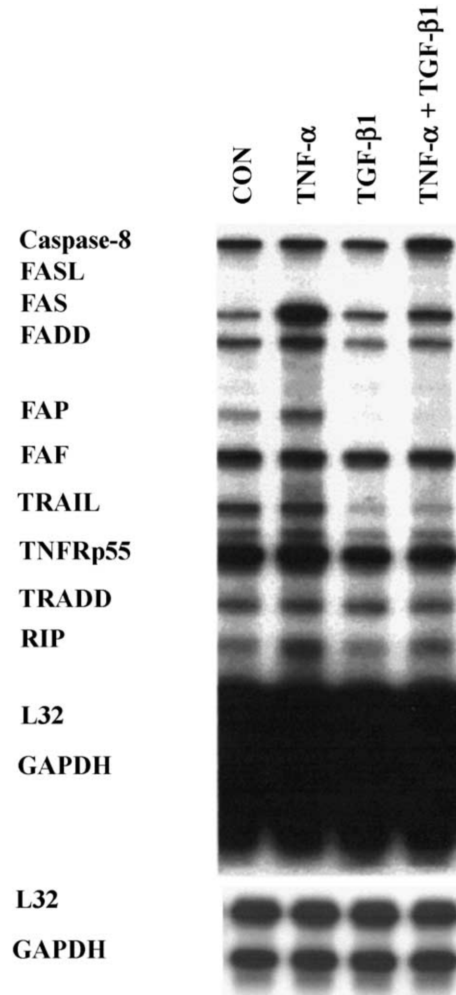

Fig. 2. Message levels of TNF-receptor family proteins in MC3T3-E1 cells. RNA was extracted from MC3T3-E1 cells treated with TNF-α, TGF-β1, or both, for 6 h. RPA was performed with mAPO3 template set as described in the Materials and methods. Lower panel shows shorter exposure of L32 and GAPDH bands. The results are representative of six separate experiments.

Table 1
Fold induction of mRNA levels

	TNF-α	TGF-β1	TNF-α + TGF-β1
Caspase-1	2.7 ± 0.3	1.1 ± 0.2[a]	1.1 ± 0.2[b]
Caspase-7	1.7 ± 0.1	1.0 ± 0.1[a]	1.0 ± 0.2[b]
Caspase-11	5.6 ± 0.8	1.8 ± 0.3[a]	3.7 ± 0.5[b,c]
Caspase-12	2.2 ± 0.2	1.0 ± 0.2[a]	1.0 ± 0.1[b]
FAS	4.0 ± 1.2	1.3 ± 0.2[a]	2.7 ± 1.1[b]
FAP	2.3 ± 0.3	0.8 ± 0.3[a]	0.8 ± 0.2[b]

Fold induction of mRNA was determined by scanning the pixel intensity of each band vs. the control level. GAPDH mRNA band was used to normalize the differences in loading. Data represent mean ± SE of six samples.

[a] $P < 0.05$, TNF-α vs. TGF-β1.

[b] $P < 0.05$, TNF-α vs. TNF-α + TGF-β1.

[c] $P < 0.05$, TGF-β1 vs. TNF-α + TGF-β1.

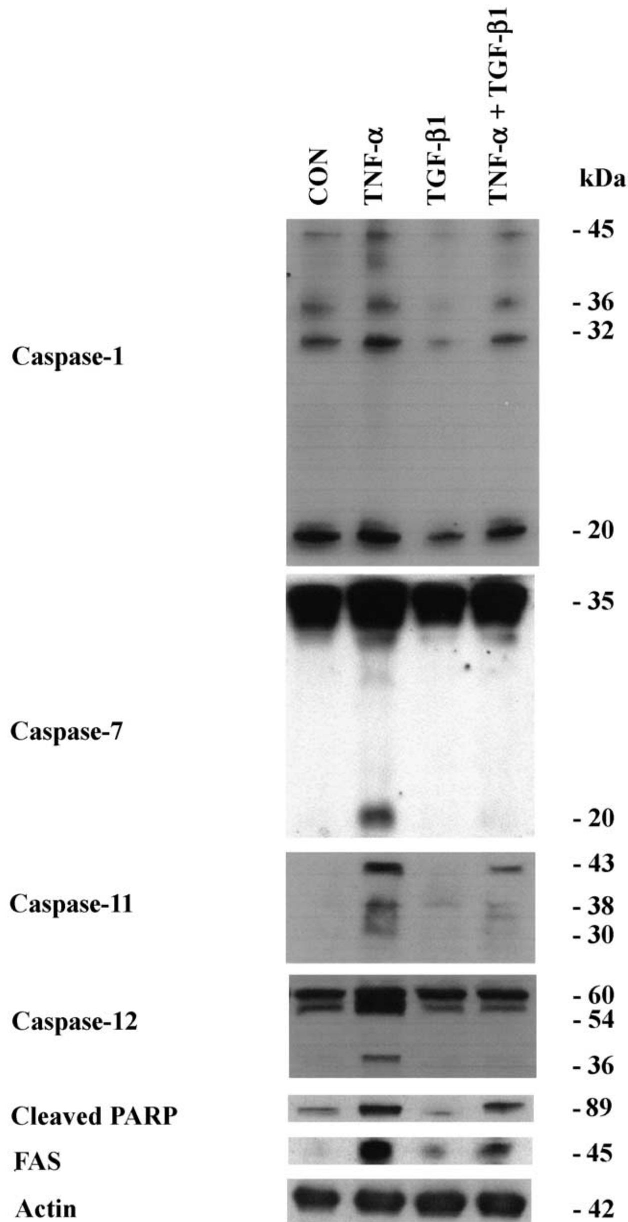

Fig. 3. Western blot analysis of apoptotic proteins. MC3T3-E1 cells were treated with or without TNF-α, TGF-β1, or both, for 24 h. Western blot analysis was carried out with antibodies specific for caspase-1, -7, -11, -12, cleaved PARP, FAS, and actin. Antibody against actin demonstrates equal loading of proteins. Data represent three separate experiments.

out by DNase I treatment and by omitting TdT from the labeling mix, respectively.

2.7. Cell death ELISA

Quantitative analysis of apoptosis was performed with a Cell Death ELISA^{Plus} kit (Roche, Indianapolis, IN). Cells (3×10^4) were plated in 48-well dishes, changed to α-MEM containing 0.5% FBS for 16 h, and treated with TNF-α, TGF-β1, or both, for 24 h. Cells were lysed with 0.2-ml lysis buffer at room temperature for 45 min. Quantities of histone-associated DNA fragments (mononucleosomes and oligonucleosomes) were determined at 405 nm with a microplate ELISA reader.

2.8. Statistical analysis

Statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison test to show differences between means. Data were represented as means ± SE. $P < 0.05$ was considered significant.

3. Results

Our first set of experiments was designed to study levels of mRNA transcripts of numerous apoptosis-regulating genes by RPA. Three commercially available gene templates were obtained from BD Pharmingen: mAPO-1 (caspase family), mAPO-2 (Bcl-2 family), and mAPO-3 (TNF-receptor family). Treatment of MC3T3-E1 cells with TNF-α for 6 h upregulated the mRNA levels of caspase-1, -7, -11, and -12, whereas the mRNA levels of caspase-2, -3, -6, -8, and -14 remained unchanged (Fig. 1). When TGF-β1 was added in combination with TNF-α, this induction was decreased. In addition to its effects on the aforementioned caspases, TNF-α also significantly upregulated the mRNA levels of FAS and FAP (Fig. 2). This upregulation, as with the caspases, was suppressed by TGF-β1 (Fig. 2). FADD, TRAIL, and RIP mRNA levels were only slightly increased by TNF-α, whereas FAF, TNFRp55, and TRADD levels did not change. In these experiments, L32 and GAPDH served as loading controls. Table 1 summarizes a quantitative analysis of the magnitude of induction for these four caspases, FAS and FAP. Neither TNF-α nor TGF-β1 had

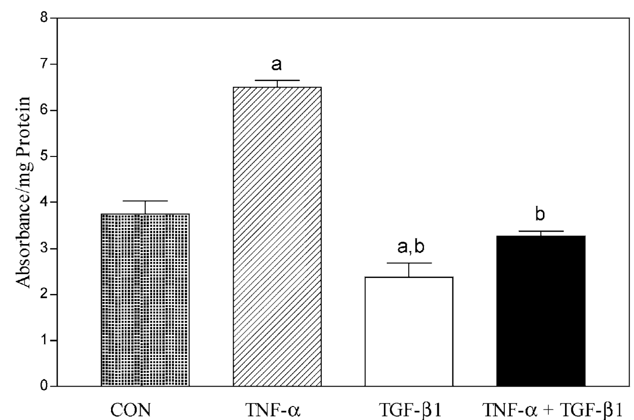

Fig. 4. Effects of TNF-α or TGF-β1 on caspase-3-like activity in MC3T3-E1 cells. Caspase-3-like activity was measured by the proteolytic cleavage of colorimetric substrate Ac-DEVD-pNA as described in the Materials and methods. Experiments were carried out three separate times. Data represent mean ± SE of four samples. a = $P < 0.05$ vs. CON, b = $P < 0.05$ vs. TNF-α treatment.

any apparent effect on the transcript levels of mAPO2 genes, including bcl-w, bfl 1, bcl-x, bak, bax, bcl-2, and bad (results not shown).

To investigate the expression of caspases at the protein level, lysates were prepared from cells treated with TNF-α, TGF-β1, or both, for 24 h. Caspase-1 was present as 45-, 36-, 32-, and 20-kDa bands in untreated cells. After 24-h exposure to TNF-α, all bands were enhanced in intensity. The smaller fragments of 36, 32, and 20 kDa represented the cleavage products of 45-kDa procaspase-1 (Fig. 3). All fragments were decreased when TGF-β1 was added along with TNF-α. In addition, procaspase-7 was detected as a 35-kDa band in untreated cells. After TNF-α treatment, the 35-kDa band increased in intensity with a concomitant appearance of the activated 20-kDa large subunit. As with caspase-1, TGF-β1 attenuated the activation of caspase-7 (Fig. 3).

Procaspase-11 was barely detectable in untreated cells. After TNF-α treatment, the two procaspase-11 bands (43 and

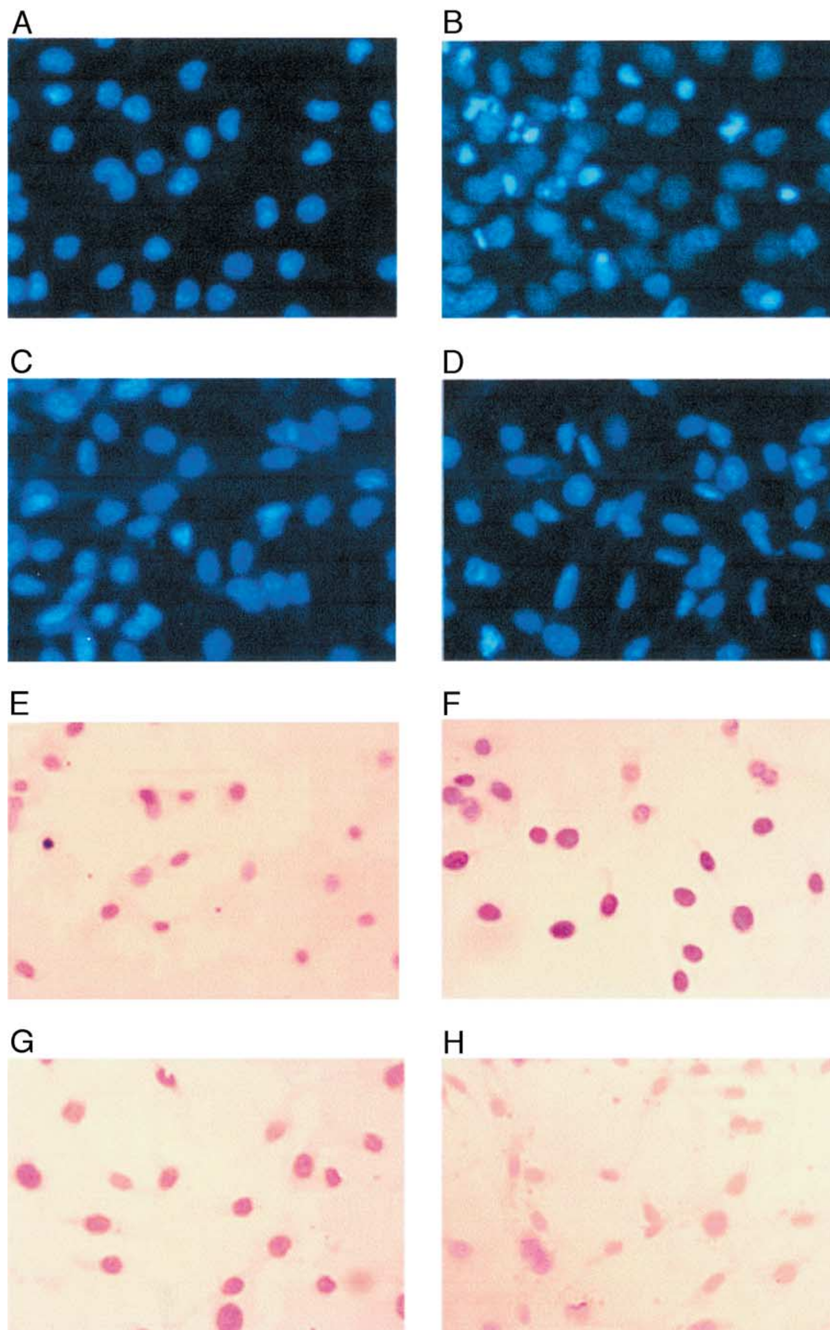

Fig. 5. Nuclear staining by Hoechst stain and identification of DNA fragmentation by TUNEL assays. MC3T3-E1 cells were treated with or without TNF-α, TGF-β1, or both, for 24 h. Panels A–D show Hoechst staining patterns of cells from (A) control, (B) TNF-α , (C) TGF-β1, and (D) TNF-α + TGF-β1. Panels E–H represent TUNEL staining patterns of cells from (E) control, (F) TNF-α, (G) TGF-β1, and (H) TNF-α + TGF-β1. TUNEL-positive nuclei were stained blue. These experiments were carried out three times.

38 kDa) appeared along with a 30-kDa fragment. In the presence of TNF-α and TGF-β1, these two procaspase bands and the 30-kDa fragment decreased in intensity (Fig. 3). Caspase-12 was present as 60- and 54-kDa bands in untreated cells. Upon TNF-α treatment, these two bands were intensified with a concomitant appearance of a 36-kDa band. The addition of TGF-β1 abrogated the induction (Fig. 3).

Poly(ADP–ribose) polymerase is a substrate for caspase-3 and -7 [24] and cleaved PARP has been shown to be an important marker for apoptosis [25]. We next determined PARP levels in cell lysates with a monoclonal antibody that was specific to the 89-kDa cleaved PARP. TNF-α treated cells had a marked increase of cleaved PARP, while the addition of TGF-β1 suppressed its level (Fig. 3). FAS, a member of the TNF receptor family, is a 45-kDa membrane protein [24]. FAS was strongly induced by TNF-α and the induction was diminished in the presence of TGF-β1 (Fig. 3).

Our RPA results did not show any change of caspase-3 mRNA level (Fig. 1). Since caspase-3 is a ubiquitous caspase that is often activated during apoptosis, we set out to examine if caspase-3 activity was elevated as a result of TNF-α treatment. Caspase-3-like activity was measured by the proteolytic cleavage of colorimetric substrate Ac-DEVD-pNA. Fig. 4 shows caspase-3-like protease activity was significantly increased 24 h after TNF-α treatment, whereas TGF-β1 could attenuate the activation. It is noted that caspase-3-like activity in TGF-β1-treated cells was lower than that of the control cells. Western blot analysis showed an induction of active caspase-3 by TNF-α and inhibition by TGF-β1. However, this induction was not as obvious as it was with caspase-1, -7, -11, and -12 (results not shown).

Since the gene template used in our RPA studies (Fig. 1) did not contain caspase-9, Western blot analysis was carried out with an antibody specific to cleaved caspase-9 to examine if TNF-α affected the processing of caspase-9. Our results showed no changes of caspase-9 pattern as a result of TNF-α or TGF-β1 treatment (data not shown).

Experiments were performed to determine if TGF-β1 could attenuate TNF-α-induced apoptosis in murine osteoblasts. DNA fragmentation was assessed by three methods: Hoechst staining, TUNEL assay, and cell death ELISA. Hoechst staining of cells treated with TNF-α showed typical pyknotic fragmented nuclei and apoptotic bodies (Fig. 5, panel B). In addition, cells treated with TGF-β1 showed few condensed nuclei (panel C), and the addition of TGF-β1 along with TNF-α reduced the number of apoptotic nuclei (panel D). Similar results were obtained with TUNEL assays. Treatment with TNF-α resulted in an extensive increase in the number of TUNEL-positive blue nuclei (panel F), while combined treatment of TGF-β1 and TNF-α significantly reduced the number of TUNEL-positive nuclei (panel H).

Finally, quantitation of apoptosis was performed by an oligonucleosomal cell death ELISA assay. As shown in Fig. 6, TNF-α increased cell death by 2.7-fold, an increase that was attenuated by the addition of TGF-β1. In this assay, we also noted that the oligonucleosomal formation in TGF-β1-treated cells was lower than the control.

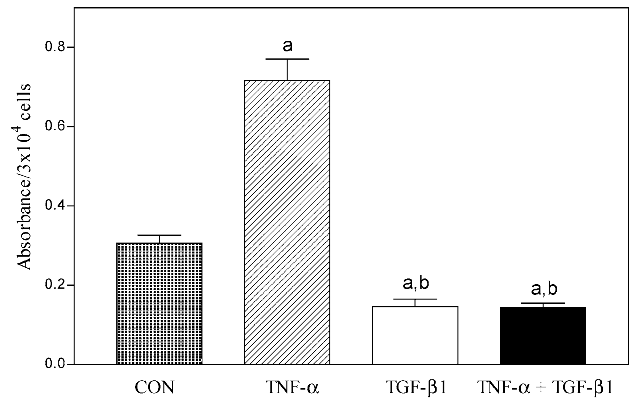

Fig. 6. Quantitative analysis of DNA fragmentation. MC3T3-E1 cells in 48-well dishes were treated with TNF-α, TGF-β1, or both, for 24 h. Cell lysates were subjected to Cell Death ELISA assay. Oligonucleosomes were measured at 405 nm by a microplate reader. Experiments were carried out three separate times. Data represent mean ± SE of eight samples. a = $P<0.05$ vs. CON, b = $P<0.05$ vs. TNF-α treatment.

4. Discussion

In this study, we show that TNF-α induces apoptosis in murine osteoblastic MC3T3-E1 cells by upregulating FAS expression and by activating caspase-1, -3, -7, -11, and -12. Furthermore, we also present evidence that TGF-β1 can decrease the apoptotic process by attenuating the expression of these apoptotic proteins. These findings support the work of Jilka et al. [12] who previously reported that TGF-β1 and interleukin-6 were able to inhibit TNF-α-induced apoptosis in murine osteoblastic MC3T3-E1 cells. However, our study expands on this previous work by exploring the involvement of various caspases pertaining to the effects of TNF-α and TGF-β1 on apoptosis.

FAS–FAS ligand system is a major pathway for the induction of apoptosis [26]. Our RPA results showed that the addition of TNF-α led to an upregulation of FAS mRNA expression by fourfold (Fig. 2) as well as the induction of FAS protein (Fig. 3), suggesting that TNF-α-induced apoptosis is partly mediated by the upregulation of FAS. Our results agreed with previous reports that TNF-α induces FAS in human osteoblasts [27,28].

Caspase-1, -4, -5, -11, -12, and -13 belong to caspase-1 subfamily. The present study shows that TNF-α upregulates three of the six members of the caspase-1 subfamily: caspase-1, -11, and -12. Caspase-1, which functions both as an initiator caspase and as an effector caspase, is the best characterized enzyme in this subfamily and is known to play a major role in inflammation and immune regulation in addition to apoptosis [29,30].

Our results demonstrated that TNF-α treatment induced the expression of caspase-11 mRNA 5.6-fold (Fig. 1). These results support previous studies that caspase-11 is crucial for apoptosis. Wang et al. [31] showed that caspase-11 knockout mice had impaired production of interleukin-1β and caspase-1 mediated apoptosis, indicating that caspase-11 is important for both inflammation and apoptosis. In addition to its role in activating caspase-1, caspase-11 also activates caspase-3 under pathological conditions [32].

To our knowledge, we demonstrate for the first time that caspase-12 is upregulated by TNF-α in osteoblasts. Procaspase-12 is predominantly localized at the ER and is activated by ER stress such as disruption of ER calcium homeostasis or accumulation of excess protein in ER [33]. Yoneda et al. [34] reported that caspase-12 is activated through a TNF-receptor-associated factor 2 (TRAF-2)-dependent mechanism in response to ER stress. Since caspase-12 is a newly discovered enzyme, its relationship with other caspases is not clear. It remains to be determined whether caspase-12 functions as an initiator caspase, an effector caspase, or both.

A unique finding of this study is that both caspase-3 and caspase-7 are specifically activated by TNF-α in osteoblasts. Caspase-7 shares 54% sequence homology with caspase-3, and it can be activated by caspase-3, -8, and -10 [35]. Caspase-7 and caspase-3 are effector caspases that are responsible for cleaving nucleases in addition to cellular substrates including lamins and PARP. A recent study has indicated that caspase-7 is also required for the activation of caspase-12 as a result of ER stress [36].

It should be noted that TGF-β1 treated cells had lower caspase-3-like activity (Fig. 4) and DNA fragmentation than the control cells (Fig. 6). It is possible that the control cells produce TNF-α and the inhibitory effects of TGF-β1 alone on apoptosis might be due to an autocrine loop of TNF-α. The inhibition of caspase production by TGF-β1 could be mediated by induction of inhibitors of apoptosis (IAPs) or anti-apoptotic Bcl2 family members. Alternatively, TGF-β1 may activate PI3K-Akt pathways. The exact mechanism awaits further investigation.

The protective role of TGF-β1 has been demonstrated in a number of studies. For example, TGF-β1 has been shown to protect mature osteoblasts from apoptosis [37]. Recently, TGF-β2, a growth factor similar to TGF-β1, was found to inhibit apoptosis in primary human osteoblasts or immortalized human osteoblasts [38]. In addition, TGF-β1 inhibits FAS-mediated apoptosis in T lymphocytes and rheumatoid synovial cells [39]. The protective mechanism of TGF-β1 warrants further investigation.

In summary, we found that TNF-α induced apoptosis by upregulating caspase-1, -3, -7, -11, -12, and FAS in MC3T3-E1 cells; these effects can be attenuated by the addition of TGF-β1. TGF-β1 may eventually be utilized as a potential therapeutic agent to treat TNF-α-induced osteoporosis in rheumatoid arthritis and periodontal disease.

Acknowledgements

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