Suppression of Tumor Necrosis Factor-mediated Apoptosis by Nuclear Factor κB-independent Bone Morphogenetic Protein/Smad Signaling*

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The activation of nuclear factor κB (NF-κB) plays a pivotal role in the regulation of tumor necrosis factor (TNF)-mediated apoptosis. However, little is known about the regulation of TNF-mediated apoptosis by other signaling pathways or growth factors. Here, unexpectedly, we found that bone morphogenetic protein (BMP)-2 and BMP-4 inhibited TNF-mediated apoptosis by inhibition of caspase-8 activation in C2C12 cells, a pluripotent mesenchymal cell line that has the potential to differentiate into osteoblasts depending on BMP stimulation. Utilizing both a trans-dominant IκBα inhibitor of NF-κB expressed in C2C12 cells and IκB kinase β-deficient embryonic mouse fibroblast, we show that BMP-mediated survival was independent of NF-κB activation. Rather, the antiapoptotic activity of BMPs functioned through the Smad signaling pathway. Thus, these findings provide the first report of a BMP/Smad signaling pathway that can inhibit TNF-mediated apoptosis, independent of the prosurvival activity of NF-κB. Our results suggest that BMPs not only stimulate osteoblast differentiation but can also promote cell survival during the induction of bone formation, offering new insight into the biological functions of BMPs.

NF-κB is a stress-responsive transcription factor that plays important roles in development and immunity (1–3). Classical NF-κB is a heterodimer composed of p50 and p65/RelA, which is sequestered in the cytoplasm by the IκB group of inhibitory proteins. Proinflammatory cytokines such as tumor necrosis factor (TNF) activate IκB kinase (IKK) complex to phosphorylate the conserved N-terminal region of IκB proteins (1–3). The phosphorylated IκB is ubiquitinated and subsequently degraded by the 26S proteasome. This results in the nuclear translocation of NF-κB and binding to NF-κB-responsive elements, followed by NF-κB-dependent transcriptional activation (1–6).

We and others have demonstrated that NF-κB plays a critical role in inhibition of TNF-mediated apoptosis (7–9). In the absence of NF-κB activation, TNF can trigger the caspase cascade by interacting with Fas-associated death domain protein, which then recruits and activates caspase-8. (11–13). Active caspase-8 promotes cell death by either directly processing other downstream caspases or cleaving the cytosolic Bid protein, a proapoptotic family member of Bcl-2 (12, 14, 15). Truncated Bid translocates to mitochondria, resulting in the release of cytochrome c from mitochondria into the cytosol and the subsequent activation of apoptosis (14, 15). However, in the presence of NF-κB activation, the caspase-8-mediated apoptotic pathway is suppressed (4). Several important antiapoptotic molecules have been identified that are transcriptionally regulated by NF-κB. These molecules include the Bcl-2 family members A1 and Bcl-xL, inhibitors of apoptosis family proteins, TNF receptor-associated family proteins, IEX-1L, and the recently elucidated NF-κB-inducible death effector domain-containing protein (4, 16–19). Although several growth factors have been found to inhibit TNF-mediated apoptosis through the activation of NF-κB (20), little is known regarding the regulation of TNF-mediated apoptosis by NF-κB-independent signaling.

Bone morphogenetic proteins (BMPs), members of the transforming growth factor β superfamily, were originally identified by their unique ability to induce bone formation in vivo (21–23). BMPs initiate a signaling cascade through the ligand-dependent activation of a complex of heteromeric transmembrane serine-threonine kinase receptors, type I and type II (24). The activated BMP type I receptor phosphorylates Smad1 and Smad5, resulting in their dissociation from the receptor complex. The phosphorylated Smad1 and Smad5 then form heterooligomeric complexes with Smad4 and translocate into the nucleus to activate the transcription of target genes (25–27). Interestingly, the BMP/Smad signaling pathway is also negatively regulated by a structurally and functionally divergent Smad protein of the subfamily of inhibitory Smads, Smad6 and Smad7 (25–27).

BMPs induce osteoblast differentiation of mesenchymal cells and are involved in postnatal bone remodeling (28, 29). Several studies demonstrate that BMPs control digit numbers in the limbs, possibly through the induction of apoptosis in the interdigital and anterior tissue (30, 31). BMPs can inhibit proliferation and induce apoptosis of multiple myeloma cells, indicating the therapeutic potential of BMPs in cancer treatment (32).
Given that the cell death machinery can be modulated by multiple factors or signaling pathways, we hypothesized that NF-κB-independent mechanisms may exist to suppress TNF-mediated apoptosis. During the search for regulators of TNF-mediated apoptosis, we unexpectedly found that in contrast to previous studies implicating BMPs in proapoptotic mechanisms (31, 32), BMPs possessed a novel antiapoptotic activity that promoted survival of mesenchymal cells during BMP-induced osteoblast differentiation. The results reported here reveal a unique BMP/Smad signaling pathway that suppresses TNF-mediated apoptosis in a NF-κB-independent manner. Given the fact that apoptosis plays an important role in osteoporosis and other inflammatory-related bone disorders (33), our results suggest that the utilization of BMP for bone regeneration and repair may have dual benefits: stimulation of osteoblast differentiation, and inhibition of apoptosis.

EXPERIMENTAL PROCEDURES

Cell Culture—C2C12 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 15% heat-inactivated fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37 °C under 5% CO2, and at 95% humidity. IKd2−/− mouse embryonic fibroblasts were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum.

Trypan Blue Exclusion, Cell Death Enzyme-linked Immunosorbent Assay, Annexin V Staining, and EGFP Survival Assay—Cells (105) were plated onto 6-well plates the day before stimulation. Cells were pretreated with the indicated concentrations of BMP-2 or BMP-4 for 2 h and subsequently killed with tumor necrosis factor (20 ng/ml) for 24 h. Cell viability was determined by trypan blue exclusion. The supernatant was collected, and cell death enzyme-linked immunosorbent assay was performed as described previously (Roche Molecular Biochemicals).

For annexin V staining, 2 × 105 cells were plated on a microscope coverslip in 24-well plates the day before stimulation. Sixteen h after treatment, cells were gently washed once with 1× binding buffer and stained with annexin V conjugated with EGFP solution (1:40; CLONTECH) and propidium iodide (50 ng/ml, Sigma) for 15 min at room temperature in the dark. After staining, cells were washed twice with 1× binding buffer and fixed in 2% formaldehyde in phosphate-buffered saline (pH 7.4) for 20 min. The coverslips were inverted on a drop of Vectashield mounting media (Fisher Scientific) on slides and examined and photographed under a fluorescence microscope using a filter set for fluorescein isothiocyanate.

For EGFP survival assay, cells were co-transfected with pCMV-GFP vector and either pCMV-Smad-7 or control vector with Superfect (Qiagen). Twenty-four h after transfection, cells were pretreated with BMP-2 or BMP-4 for 2 h and then killed with TNF for an additional 24 h. The green fluorescent protein-expressing cells were examined directly by a fluorescence microscope.

Western Blot Analysis—Cells (2 × 106) were plated in a 100-mm plate the day before stimulation. Cells were pretreated with BMP-2 or BMP-4 at a concentration of 200 ng/ml for the indicated times. The detached and attached cells were collected. Whole cell extracts were prepared with radioimmunoprecipitation buffer containing 1% Nonidet P-40, 5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 100 mM sodium orthovanadate, and a 1:100 mixture of protease inhibitors (Sigma). The proteins were resolved in SDS-10% polyacrylamide gels by electrophoresis and transferred to polyvinylidene difluoride membrane by semidy transfer apparatus (Bio-Rad). The membrane was probed with polyclonal antibodies against caspase-8 (Santa Cruz Biotechnology) and visualized using ECL reagent (Amershame Pharmacia Biotech) according to the manufacturer’s instructions (10).

In Vitro Caspase-3 and Caspase-8 Activity Assay—Cells were treated as described above for Western blot. The detached and attached cells were collected, washed with phosphate-buffered saline, and lysed in 200 μl of ice-cold hypotonic lysis buffer provided by the manufacturers (R&D Systems, CLONTECH, or Promega). The cell extracts were centrifuged, and supernatants were collected. Protein extracts (200–300 μg) were incubated in reaction buffer containing IETD-pNA (colorimetric caspase-8 substrate; R&D Systems) or DEVD-pNA (colorimetric caspase-3 substrate; Promega) at 37 °C for 2 to 3 h. The samples were analyzed with a plate reader by measuring the optical density at a wavelength of 405 nm.

Electrophoretic Mobility Shift Assays and NF-κB Luciferase Reporter Assays—Cells were treated with TNF (10 ng/ml) for the indicated time points. Nuclear extracts were prepared for electrophoretic mobility shift assays as described previously (15, 22). Aliquots (5 μg) of nuclear extracts were preincubated with 1 μg of poly(dI-dC) in binding buffer (10 mM Tris, pH 7.7, 50 mM NaCl, 20% glycerol, 1 mM dithiotheritol, and 0.5 mM EDTA) for 10 min at room temperature. Approximately 20,000 cpm of 32P-labeled DNA probe containing the class 1 major histocompatibility complex NF-κB site (5′-CAGGGGCTGGGGATTCCACAtTCGACAATCTAATC-3′) was then added, and binding proceed for 15 min. The complexes were separated on a 5% polyacrylamide gel and exposed for autoradiography (5, 17).

To determine NF-κB transcription activity, cells were transfected with 2× IκB-dependent luciferase reporter constructs using the Superfect reagent (Qiagen) according to the manufacturer’s instructions (20). PRL-TK Renilla luciferase reporter was co-transfected to normalize for transfection efficiency. Luciferase activities were measured using a dual luciferase system (Promega).

RESULTS

BMP-2 and BMP-4 Inhibit TNF-mediated Apoptosis—C2C12 cells are pluripotent mesenchymal cells that are widely used for the study of cell differentiation in vitro (6, 28). To study the role of NF-κB in cell growth and apoptosis, we have established a C2C12 cell line stably expressing a modified form of the NF-κB inhibitor, super-repressor-IκBα (SR-IκBα; Fig. 1A). SR-IκBα contains serine-to-alanine mutations at residues 32 and 36, which prevent signal-induced phosphorylation and subsequent proteasome-mediated degradation of IκBα. As shown in Fig. 1A, TNF rapidly induced the phosphorylation and degradation of endogenous IκBα in C2C12 control cells (C2C12V) but

![Image](321x491 to 542x730)
did not induce phosphorylation and degradation of SR-IκBα in C2C12I cells. As predicted, SR-IκBα inhibited the nuclear translocation of NF-κB induced by TNF (Fig. 1). Consistent with our previous studies (10), C2C12I cells were sensitive to TNF-mediated killing, confirming that NF-κB inhibits TNF-mediated apoptosis in cells (Fig. 2A).

Given the fact that the cell death machinery could be modulated by different factors and stimuli, we hypothesized that a NF-κB-independent pathway might be able to regulate TNF-mediated apoptosis. Thus, we utilized C2C12I cells to screen new growth factors or signaling molecules that could regulate TNF-mediated apoptosis in a NF-κB-independent fashion. Because C2C12 cells are a well-established model system to study BMP-induced osteoblast differentiation, we sought to determine whether BMPs would modulate TNF-mediated apoptosis. Previously, Lopez-Rovira et al. (34) have found that transforming growth factor β activates NF-κB that is independent of IκB degradation. Thus, to confirm the observation that BMPs did not activate NF-κB in C2C12I cells, NF-κB-dependent luciferase reporter assays were performed. As shown in Fig. 1C, BMP-4 did not induce NF-κB in C2C12I cells, NF-κB-dependent luciferase reporter assays were performed. As shown in Fig. 1C, BMP-4 did not induce NF-κB transcriptional activities in C2C12I cells. To test whether BMPs promoted cell survival, C2C12I cells were pretreated with BMP-4 or vehicle control for 2 h and then treated with TNF for 24 h. As shown in Fig. 2A, trypan blue exclusion analysis found that TNF stimulation caused 70–80% cell death in C2C12I cells. In contrast, only approximately 20% of cells were killed by TNF when cells were pretreated with BMP-4. BMP-4-mediated protection against TNF killing was dose-dependent, as shown in Fig. 2B.

Furthermore, we also examined whether another BMP family member could inhibit TNF killing. As shown in Fig. 2, C and D, BMP-2, like BMP-4, also potently inhibited TNF killing in a dose-dependent manner.

To further confirm the results described above, DNA fragmentation and histone release from cell culture supernatant were also measured with cell death enzyme-linked immunosorbent assay (4). As shown in Fig. 3, A and B, pretreatment with BMP-2 and BMP-4 significantly inhibited DNA fragmentation induced by TNF. To determine whether BMP-mediated survival was due to the modification of apoptosis, EGFP-annexin V staining of apoptotic cells. Cells were untreated or pretreated with BMP-2 (200 ng/ml) or BMP-4 (100 ng/ml) for 2 h and then killed by TNF for 14–16 h. Cells were washed and incubated with EGFP-annexin V (1:40) and propidium iodide. The cells were examined by fluorescence microscopy.

Next, we examined the molecular mechanism by which BMP signaling inhibited TNF-mediated apoptosis. To initiate apoptosis, TNF binds its receptor to recruit TNF receptor-associated death domain protein and Fas-associated death domain protein to activate caspase-8 (4, 8, 11). To determine whether BMPs inhibited TNF-induced caspase-8 activation, cells were pretreated with BMP-2 and subsequently killed by TNF. As shown in Fig. 4A, Western blot analysis

**Fig. 2.** BMP-2 and BMP-4 inhibit TNF-mediated apoptosis through a NF-κB-independent mechanism. A, BMP-4 inhibited TNF killing. Cells were pretreated or not pretreated with BMP-4 for 2 h (100 ng/ml) and then treated with TNF (20 ng/ml) for 24 h. Cell viability was determined by trypan blue exclusion. The assays were performed in triplicate, and the results represent the mean value from three independent experiments. Statistical differences between each group were determined by Student’s t test. *p < 0.01. B, BMP-4 inhibited TNF killing in a dose-dependent fashion. Cells were pretreated with the indicated concentrations of BMP-4 and then killed by TNF. C and D, BMP-2 inhibited TNF killing. The experiments were performed as described in A and B, respectively. Statistical differences between each group were determined by Student’s t test. *p < 0.01.

**Fig. 3.** BMP-2 and BMP-4 inhibit TNF-mediated apoptosis. A and B, BMP-2 and BMP-4 inhibited DNA fragmentation. Cell treatment was performed as described in the Fig. 1 legend. Cell supernatant (20 μl) from each group was incubated with anti-histone and anti-DNA antibody at room temperature for 2 h. The reaction was measured with a microplate reader at a wavelength of 405 nm. The assays were performed in triplicate, and the results represent the mean value from three independent experiments. Statistical differences between each group were determined by Student’s t test. *p < 0.01. C, annexin V staining of apoptotic cells. Cells were untreated or pretreated with BMP-2 (200 ng/ml) or BMP-4 (100 ng/ml) for 2 h and then killed by TNF for 14–16 h. Cells were washed and incubated with EGFP-annexin V (1:40) and propidium iodide. The cells were examined by fluorescence microscopy.
found that the processing of caspase-8 induced by TNF was strongly inhibited by BMP-2. Because we could not detect the active subunit of caspase-8 by Western blot analysis, we performed the caspase-8 enzymatic assay to determine whether BMP-2 suppressed caspase-8 activity induced by TNF. As shown in Fig. 4B, caspase-8 enzymatic activity induced by TNF was inhibited by BMP-2. A proapoptotic family member of Bcl-2, Bid, has been found to be a specific substrate of caspase-8 during death receptor-mediated apoptosis. The truncated Bid promotes and/or amplifies apoptosis by inducing the release of cytochrome c from mitochondria to the cytosol (14, 15). To confirm that caspase-8 activity was inhibited by BMP-2, we examined the cleavage of Bid. As shown in Fig. 4A, a substantial amount of truncated Bid was detected in cells after TNF stimulation, but not in cells pretreated with BMP-2. In addition to the cleavage of Bid, caspase-8 can also directly activate executing caspases such as caspase-3 to induce apoptosis (11, 13). Thus, we also determined whether caspase-3 activity was affected by the inhibition of caspase-8. As predicted, significantly lower DEVDase activity was present in the cells pretreated with BMP-2 compared with cells without BMP-2 pretreatment after TNF stimulation (Fig. 4C). These results demonstrated that BMP-2 suppressed TNF-mediated apoptosis by inhibiting caspase-8 activation.

**BMP-mediated Cell Survival Is Dependent on SMAD Signaling**—Because the results presented above were from C2C12 cells in which NF-κB activities were suppressed, they indicated that BMP-2- or BMP-4-mediated survival was independent of NF-κB. Biochemical and genetic studies have demonstrated that BMPs exhibit their biological functions through the Smad signaling pathway (35–37). Thus, we examined whether BMP-mediated cell survival was dependent on the Smad signaling pathway. To block BMP-mediated Smad signaling, an inhibitory Smad, Smad7, was utilized. Smad7 interacts with activated BMP type I receptors and inhibits BMP-mediated signaling by inhibiting Smad1 and Smad5 phosphorylation (37). Cells were co-transfected with pEGFP expression vector and pCMV-Smad7 expression vector or a control vector. Twenty-four h after transfection, cells were treated with TNF (20 ng/ml) for an additional 24 h. The green fluorescent protein-expressing cells were examined by fluorescence microscopy. The assays were performed in duplicate. The results represent the average value of three independent experiments. Statistical differences between each group were determined by Student’s t test. *p < 0.01. B, photograph of EGFP-positive cells. Cell transfection was performed as described above. The surviving cells (EGFP-positive cells) were examined by fluorescence microscopy.
and Bik, was not modulated by BMPs. Thus, these results suggest that BMPs function in a proapoptotic manner perhaps required for interdigit cell death (30, 31). BMPs have been found to induce apoptosis in multiple myeloma (32). These seemingly contradictory findings may be due to cell type-specific effects. However, it is of interest that both C2C12 cells and embryonic fibroblasts used for our studies have the potential to differentiate into osteoblasts upon BMP stimulation (28, 29). The process of osteoblast differentiation may be a stress stimulus that requires a survival signal to prevent the cell from undergoing apoptosis (33). Consistent with our results, Yang et al. (39) have found that deletion of Smad5 led to apoptosis in mesenchymal cells and angiogenesis defects during early mouse development by unknown mechanisms. Given the fact that Smad5 is a key component of BMP signaling (35, 36), the result implies that BMP/Smad signaling may play a role in cell survival during embryogenesis and development. In conclusion, our results suggest that BMPs can prevent apoptosis as well as stimulate osteoblast differentiation and provide new insight into biological functions of BMPs.

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