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BMP-4 and retinoic acid synergistically induce activation of caspase-9 and cause apoptosis of P19 embryonal carcinoma cells cultured as a monolayer

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Abstract

In monolayer cultures of P19 EC cells treated with both *all-trans* retinoic acid (RA) and bone morphogenetic protein (BMP)-4 (RA/BMP-4 treatment), many non-adherent apoptotic cells and activated caspase-3-positive cells were observed, but they were not observed in cells treated with RA or BMP-4 alone. Consistent with the appearance of activated caspase-3-positive cells, BMP-4 and RA together induced processing of caspase-9, Ac-DEVD-MCA cleavage activity and DNA fragmentation. These three activities were observed infrequently or not at all when cells were treated with RA or BMP-4 alone. In the RA/BMP-4 treatment-induced apoptosis, caspase-9 was upstream of caspase-3 in the enzyme cascade, and the caspase-9 to -3 step was key in the apoptotic pathway. Bcl-xL inhibited processing of caspase-9, Ac-DEVD-MCA cleavage activity and DNA fragmentation induced by RA/BMP-4 treatment. However, unlike staurosporine-induced apoptosis, cytochrome c, which activates caspase-9, was not detected in the cytosol of RA/BMP-4-treated cells. RA and BMP-4 may activate caspase-9 through an apoptotic pathway other than the Apaf-1/cytochrome c pathway. The prominent decrease of X-chromosome-linked inhibitory apoptosis protein (XIAP) in the cytosol may explain the activation of caspase-9 induced by RA and BMP-4 treatment.

Keywords: P19 EC cells; caspase-9; BMP-4; retinoic acid; XIAP

Abbreviations: BMP-4, bone morphogenetic protein-4; RA, *all-trans* retinoic acid; XIAP, X-chromosome-linked inhibitor of apoptosis protein

Introduction

During development, bone morphogenic protein (BMP)-4, a member of the TGF-β family, induces apoptosis of cells in the interdigits[1] and of neural crest-derived cells located in the rhombomeres.[2,3] Disruption of BMP-2 and -4 signaling by a dominant-negative BMP type I receptor mutation prevents interdigital apoptosis.[4] Similarly, mutant mice with a targeted deletion of the retinoic acid receptor (RAR) α gene and one or both alleles of the RAR γ gene show a marked decrease in the number of apoptotic cells in the interdigital necrotic zones.[5] Thus, both retinoic acid and BMP signals are involved in apoptosis of cells in the interdigits.

All-trans retinoic acid (RA) induces neuroectodermal differentiation in aggregated P19 EC cells and mesoderm and endoderm differentiation in P19 EC cell monolayers.[6] During RA-induced neuronal differentiation of P19 EC cells, many cells undergo cell death with the hallmarks of apoptosis, including cytoplasmic contraction and DNA fragmentation.[7,8] BMP-4 alone and RA alone cause slight induction of apoptosis of P19 EC cells cultured as monolayers, but BMP-4 and RA together induce severe apoptosis of P19 EC cells.[9,10] RA/BMP-4-induced apoptosis is mediated through activation of RAR α and γ in P19 EC cells.[10] However, little is known about the molecular mechanism by which BMP-4 and RA synergistically induce apoptosis.

Genetic studies of apoptosis in *Caenorhabditis elegans* have identified genes involved in apoptosis: *ced-9* prevents apoptosis[11] and *ced-3* and *ced-4* promote apoptosis.[12,13] In mammals, caspases, which are homologues of Ced-3, are involved in various types of cell death.[14,15] Caspases are activated by a cascade of sequential enzymatic cleavages by other members of this family.[16] During apoptosis, caspase-3, which is the last enzyme in this cascade, is activated by processing of procaspase-3 (p32) into its active forms (p20/17 and p12).[17] Caspase-9, which is activated through its association with Apaf-1, a homologue of Ced-4 that also binds cytochrome c,[18] is autoprocessed into its active form, and it activates caspase-3.[19]

Recently, several lines of evidence have suggested that caspases are involved in BMP-mediated cell death. Caspase inhibitors prevent apoptosis of cells in the interdigits of limbs,[20,21] where RA and BMP signals cause apoptosis. Using a cleavage site-directed antibody against caspase-3 (anti-p20/17), we previously demonstrated that activated caspase-3-positive cells are located in the interdigits of the limbs.[22] TAK1, a member of the MAP kinase kinase kinase family that functions in the TGF-β/BMP signaling pathway,[23] induces apoptosis in *Xenopus* embryos.[24] Bcl-2, a homologue of Ced-9, blocks TAK1-induced apoptosis. X-chromosome-linked inhibitor of

apoptosis protein (XIAP),[25] which can directly inhibit caspase activity,[26] has been shown to bind TAB1, an activator of TAK1, and inhibit TAK1-induced apoptosis.[27] Furthermore, BMP-2-induced neuronal cell death is inhibited by wide range caspase inhibitor z-VAD.[28]

In the present study, we demonstrate that BMP-4 and RA synergistically decrease XIAP in the cytosol of P19 EC cells cultured as monolayers and activate the caspase-9 to -3 apoptotic pathway.

Results

We used the anti-p20/17 antibody to examine immunohistochemically whether caspase-3 is activated when apoptosis is induced in P19 EC cells by RA- and/or BMP-4 treatment. In monolayer cultures of P19 EC cells treated for 24 h with RA and BMP-4 together, many non-adherent cells demonstrating cytoplasmic contraction, a hallmark of apoptosis, and many p20/17-positive cells were observed, but they were observed infrequently or not at all in the cultures incubated with RA or BMP-4 alone (Figure 1).

Consistent with the appearance of p20/17-positive cells, Ac-DEVD-MCA cleavage activity, which reflects activation of caspase-3, increased when cells were incubated with RA and BMP-4 together for 24 h (Figure 2A). RA (1000 nM) alone induced caspase-3 activation slightly and BMP-4 (10 ng/ml) alone did not. DNA fragmentation was also induced by RA/BMP-4-treatment and corresponded to the increase in Ac-DEVD-MCA cleavage activity (Figure 2B).

We examined the caspases upstream of caspase-3 that are activated during apoptosis induced by RA/BMP-4-treatment. Caspase-9 is one of the enzymes upstream of caspase-3. The molecular weight of mouse caspase-9 predicted from the nucleotide sequence is 50 kDa (nucleotide sequences have been deposited into DDBJ, Accession No. ABO19600). Only two amino acids in the peptide of mouse caspase-9 differed from those of human caspase-9, which was used as antigen to generate anti-caspase-9 (I16). When N-terminal FLAG-tagged (N-FLAG) mouse caspase-9 was transfected into COS cells, 50 kDa (p50) and 37 kDa (p37) bands were detected with both anti-FLAG and anti-caspase-9 (Figure 3A).

When N-FLAG mouse caspase-9 was transfected into P19 EC cells, Ac-DEVD-MCA activity increased in a time-dependent manner (Figure 3B). p50 was detected with anti-caspase-9 in untreated P19 EC cells, and 30 kDa (p30) bands as well as p50 bands were detected in P19 EC cells transfected with mouse caspase-9 (Figure 3C). However, no bands were detected with anti-FLAG because the N-terminal region of caspase-9 is cleaved off in P19 EC cells (Fujita, unpublished data). Ac-DEVD-CHO (10 μM), which completely inhibited Ac-DEVD-MCA cleavage activity, did not inhibit the processing of caspase-9 (p50) into p30 in P19 EC cells (Figure 3C).

In addition to the activation of caspase-3, generation of p30 was also induced by RA/BMP-4-treatment, and it corresponded to the increase in Ac-DEVD-MCA cleavage activity (Figure 4A). Furthermore, Ac-DEVD-CHO (1–20 μM) inhibited the DNA fragmentation induced by RA/BMP-4 treatment but did not affect the processing of caspase-9 (Figure 4B,C). Thus, caspase-9 is upstream of caspase-3 in the apoptosis pathway induced by RA/BMP-4-treatment.

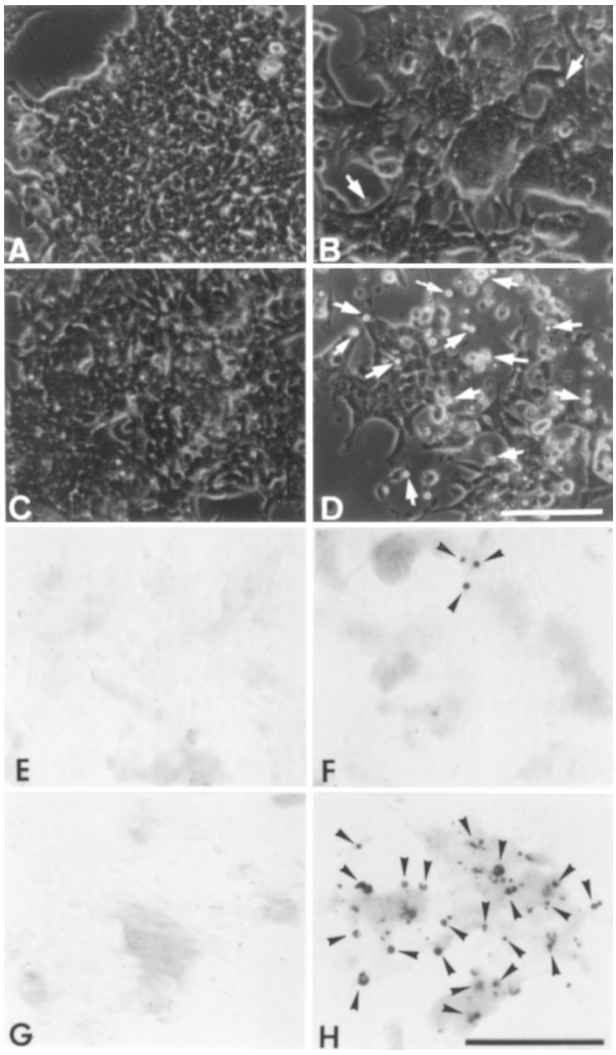

Figure 1 Appearance of apoptotic cells and activated caspase-3 (p20/17)-positive cells in the P19 EC cells cultured as monolayer with RA and BMP-4 together. After P19 EC cells were treated with RA (1000 nM) and/or BMP-4 (10 ng/ml) for 24 h, appearance of apoptotic cells and p20/17-positive cells were examined. (**A–D**) Photomicrographys of the cells observed under light microscope. (**E–H**) Immuno-cytochemical staining of activated caspase-3 (p20/17)-positive cells. (**A**,**E**) Untreated cells, (**B**,**F**) RA-treated cells, (**C**,**G**) BMP-4-treated cells, and (**D**,**H**) RA/BMP-4-treated cells. Many apoptotic cells with cytoplasmic contraction (arrows) and p20/17-positive cells (arrowheads) were detected in the RA/BMP-4-treated cells. Bars indicate 200 μm

The synergistic effects of RA and BMP-4 on processing of caspase-9 were examined. RA (100–1000 nM) slightly induced processing of caspase-9 and changed Ac-DEVD-MCA cleavage activity in monolayer cultures of P19 EC cells. BMP-4 (1 ng/ml) increased Ac-DEVD-MCA cleavage activity, processing of caspase-9, and DNA fragmentation of P19 EC cells treated with 1–1000 nM RA (Figure 5). RA (100 nM) in combination with BMP-4 (1 ng/ml) yielded the maximum effect on Ac-DEVD-MCA cleavage activity.

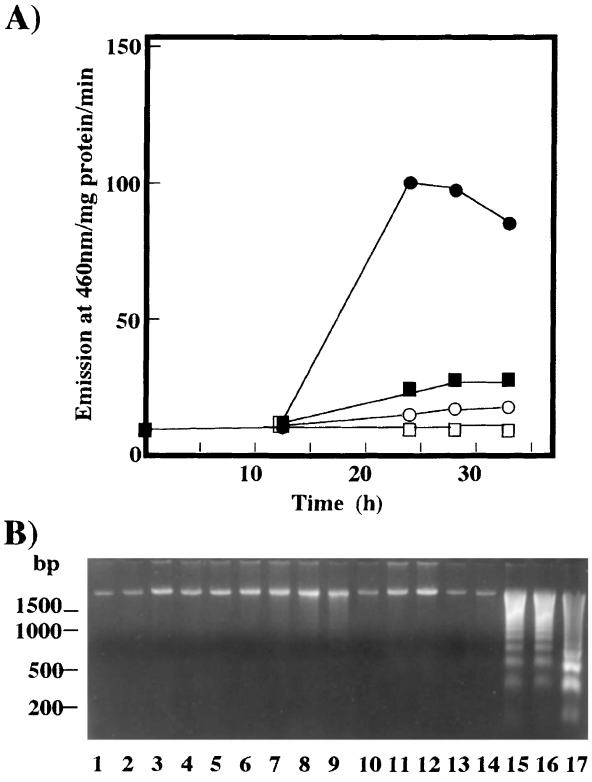

Figure 2 Time-dependent effects of RA/BMP-4 treatment on the Ac-DEVD-MCA cleavage activity and DNA fragmentation. (**A**) Ac-DEVD-MCA cleavage activity in the untreated cells (open squares), the BMP-4 (10 ng/ml)-(open circles), the RA (1000 nM)-(closed squares), and RA (1000 nM)/BMP-4 (10 ng/ml)-(closed circles) treated cells. (**B**) DNA fragmentation of RA- and/or BMP-4-treated cells. Lane 1; 0 h, lane 2–5; untreated cells, lane 6–9; RA-treated cells, lane 10–13; BMP-4-treated cells, lane 14–17; RA and BMP-4-treated cells. Lane 1; 0 h, lane 2, 6, 10, and 14; at 12 h, lane 3, 7, 11, and 15; at 24 h, lane 4, 8, 12, and 16; at 28 h, lane 5, 9, 13, and 17; at 33 h

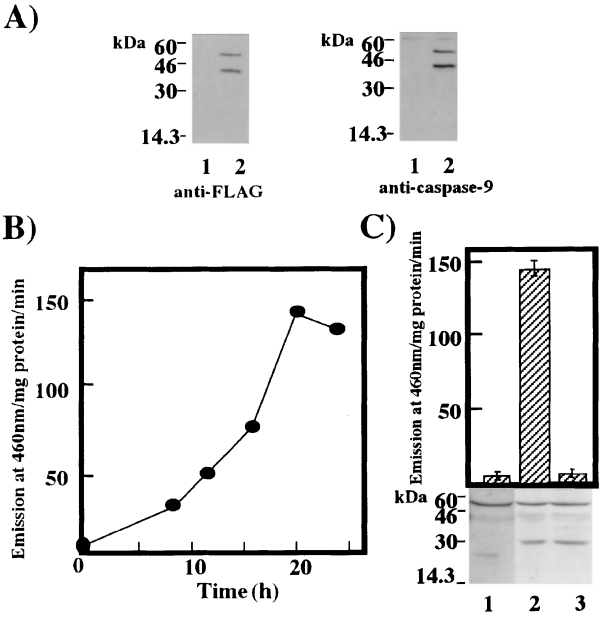

Figure 3 Autoprocessing fragment of caspase-9 inducing the activation of caspase-3. (**A**) The processing of caspase-9 overexpressed in COS cells. Mouse caspase-9 in N-FLAG-tagged pcDEF3 vector was transfected into COS cells. The processing fragments were detected by anti-FLAG and anti-caspase-9 (I16). Lane 1; untreated COS cells, lane 2; COS cells transfected with mouse caspase-9. (**B**) Time-dependent Ac-DEVD-MCA cleavage activity after P19 EC cells were transfected with caspase-9 in N-FLAG-tagged pcDEF3 vector. (**C**) Effect of Ac-DEVD-CHO on the processing of caspase-9 (lower panel) and Ac-DEVD-MCA cleavage activity (upper panel) in P19 EC cells. P19 EC cells were transfected with caspase-9 in the presence or absence of Ac-DEVD-CHO (10 μM). After 8 h, cells were washed with the medium and further incubated with or without 10 μM Ac-DEVD-CHO for 20 h. The processing of caspase-9 was detected by immunoblot analysis using anti-caspase-9 (I16). Lane 1; vector DNA transfected cells, lane 2; caspase-9 transfected cells, lane 3; caspase-9 transfected cells in the presence of Ac-DEVD-CHO

BMP-4 (0.1–5 ng/ml) alone did not induce processing of caspase-9, change Ac-DEVD-MCA cleavage activity, or induce DNA fragmentation in the monolayer cultures of P19 EC cells. BMP-4 (0.1–5 ng/ml) enhanced these changes in a dose-dependent fashion in P19 EC cells treated with 1000 nM RA (Figure 6). Thus, BMP-4 alone could not induce activation of caspase-9, but it enhanced the RA-induced activation of caspase-9 that led to the apoptosis of P19 EC cells.

Bcl-xL, a member of the Bcl-2 family that inhibits activation of caspase-3 through interaction with Apaf-1 and caspase-9,[29,30] inhibited Ac-DEVD-MCA cleavage activity and DNA fragmentation in P19 EC cells treated with both RA (1000 nM) and BMP-4 (0.1–5 ng/ml) (Figure 7A,B). Bcl-xL also inhibited processing of caspase-9 in cells treated with RA and BMP-4.

Cytochrome c released from mitochondria activates caspase-9 by association with Apaf-1 in the cytosol.[18] The levels of cytochrome c and other molecules, which regulate processing of caspase-9, were examined in the cytosol of non-adherent apoptotic cells and adherent living cells after RA/BMP-4-treatment. The non-adherent cells showed high levels of DNA fragmentation, whereas adherent cells did not (Figure 8A). Consistent with DNA fragmentation, Ac-DEVD-MCA cleavage activity in the cytosol of the non-adherent cells was approximately four times higher than that in the cytosol of all cells. Processing of caspase-9 was also preferentially stimulated in the cytosol of non-adherent cells but not in the cytosol of adherent cells (Figure 8B). However, Apaf-1 and cytochrome c levels in the cytosol of the non-adherent and adherent RA/BMP-4-treated cells matched those in the cytosol of untreated P19 EC cells. In contrast, XIAP was down-regulated in the cytosol of non-adherent cells but not in the cytosol of adherent cells. In the staurosporine-induced apoptotic cells, however, the level of cytochrome c increased in the cytosol, whereas the levels of XIAP and Apaf-1 did not change.

Discussion

Caspase cascade in the apoptosis of P19 EC cells induced by RA and BMP-4

Caspase-9 and caspase-3 were activated in the apoptosis of P19 EC cells induced by RA/BMP-4-treatment (Figures 2, 4, 5

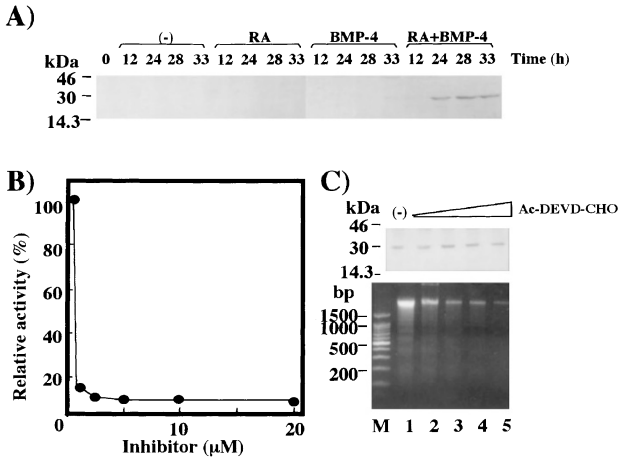

Figure 4 Caspase-9 is upstream of caspase-3 activated in the apoptosis induced by RA and BMP-4 together. (**A**) Activation of caspase-9 in RA- and/or BMP-4-treated cells. Processing of caspase-9 into p30 was examined by immunoblot analysis using anti-caspase-9 (I16). (**B**) The concentration of Ac-DEVD-CHO required to inhibit Ac-DEVD-MCA cleavage activity induced by RA and BMP-4 together. (**C**) Effect of Ac-DEVD-CHO on the activation of caspase-9 and DNA fragmentation induced by RA and BMP-4 together. Lane 1–5; processing fragment of caspase-9 (upper panel) and DNA fragmentation (lower panel) induced by RA and BMP-4 in the presence of Ac-DEVD-CHO at 0, 1, 2, 5 and 10 μM, respectively. M; size marker of DNA

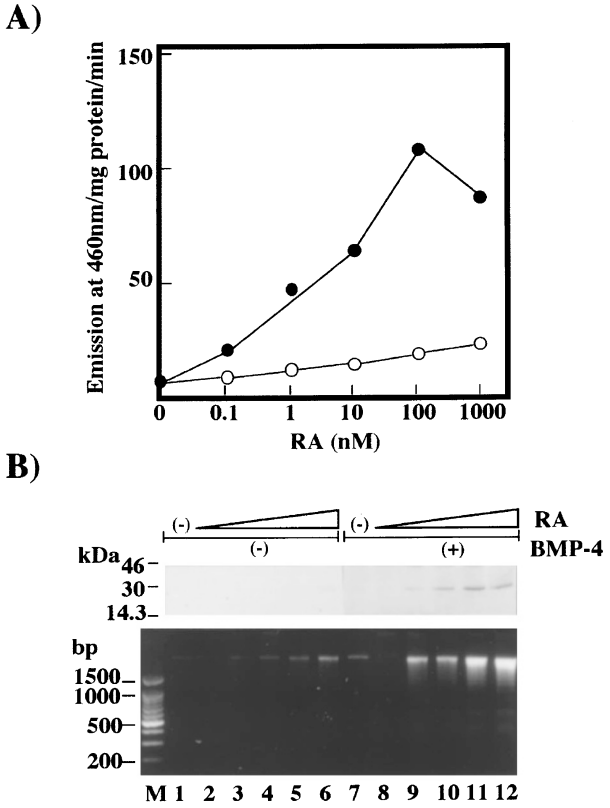

Figure 5 The RA concentration required to induce the synergistic effects on the activation of caspase-9, Ac-DEVD-MCA cleavage activity and DNA fragmentation of P19 EC cells in the cooperation with BMP-4. (**A**) Dose-dependent effects of RA on Ac-DEVD-MCA cleavage activity of P19 EC cells treated with (closed circles) or without BMP-4 (1 ng/ml) (open circles). (**B**) Dose-dependent effects of RA on the processing of caspase-9 (upper panel) and DNA fragmentation (lower panel). Lane 1 and 7; without RA, lane 2 and 8; with RA at 0.1 nM, lane 3 and 9; with RA at 1 nM, lane 4 and 10; with RA at 10 nM, lane 5 and 11; with RA at 100 nM, lane 6 and 12; with RA at 1000 nM. Lane 1–6; without BMP-4, lane 7–12; with BMP-4 (1 ng/ml). M; size marker of DNA

and 6). Caspases are activated via sequential processing by other caspases.[17] In the caspase cascade, caspase-9 is upstream of caspase-3;[31–33] caspase-3 is not activated in caspase-9-deficient mice. However, caspase-3 also participates in a feedback amplification loop involving caspase-9,[33] and the activated caspase-3 cleaves caspase-9. During apoptosis of P19 EC cells induced by RA/BMP-4-treatment, the caspase-9 to -3 apoptotic pathway was activated (Figures 3, 4). Caspase-9 induced Ac-DEVD-MCA cleavage activity in P19 EC cells, whereas Ac-DEVD-CHO inhibited DNA fragmentation but not processing of caspase-9 induced by RA/BMP-4-treatment. It is unlikely that the caspase-3 to -9 feedback pathway is involved in RA/BMP-4-mediated apoptosis.

We do not exclude the possibility that other caspases upstream of caspase-3 are also activated by RA/BMP-4. However, we could not detect changes in caspase-1, -6, -7, and -8 activities in RA/BMP-4-treated cells using synthetic substrates for caspases (data not shown). Inhibitors of these caspases did not prevent apoptosis or Ac-DEVD-MCA cleavage activity induced by RA/BMP-4-treatment (data not shown). These results indicate that the caspase-9 to -3 pathway is the predominant pathway in the apoptosis of P19 EC cells induced by RA/BMP-4-treatment. Development of a specific inhibitor of autoprocessing of caspase-9 is currently underway for further analysis.

Cytochrome c release in the RA and BMP-4-treated apoptotic cells

Bcl-xL prevented processing of caspase-9 induced by RA/BMP-4 treatment (Figure 7). At present, the only apoptotic pathway known to activate caspase-9 and to be regulated by Bcl-xL is the Apaf-1/cytochrome c-induced pathway.[29,30] Bcl-xL inhibits activation of caspase-9 through two possible mechanisms: by preventing release of cytochrome c from mitochondria or by forming a complex with Apaf-1 and caspase-9. However, unlike staurosporine,[34] RA/BMP-4 treatment did not increase cytochrome c levels even in the cytosol of non-adherent apoptotic cells with high caspase-9 processing activity and Ac-DEVD-MCA cleavage activity (Figure 8), suggesting that RA and BMP-4 signals activate caspase-9 without releasing cytochrome c from mitochondria. Recently, an alternative pathway that activates the caspase cascade was reported. RGD peptides directly activate caspases independent of the Apaf-1/cytochrome c apoptotic pathway.[35] RA and BMP-4 signals may activate caspase-9 through such an alternative pathway. However, reductions in mitochondria transmembrane potentials, which accompany early apoptosis in many situations, were detected in the RA and RA/BMP-4-treated cells as well as in staurosporine-treated cells by fluorescence of the cationic lipophilic dye CMTMRos[34] (Momoi, unpublished data). We do not exclude

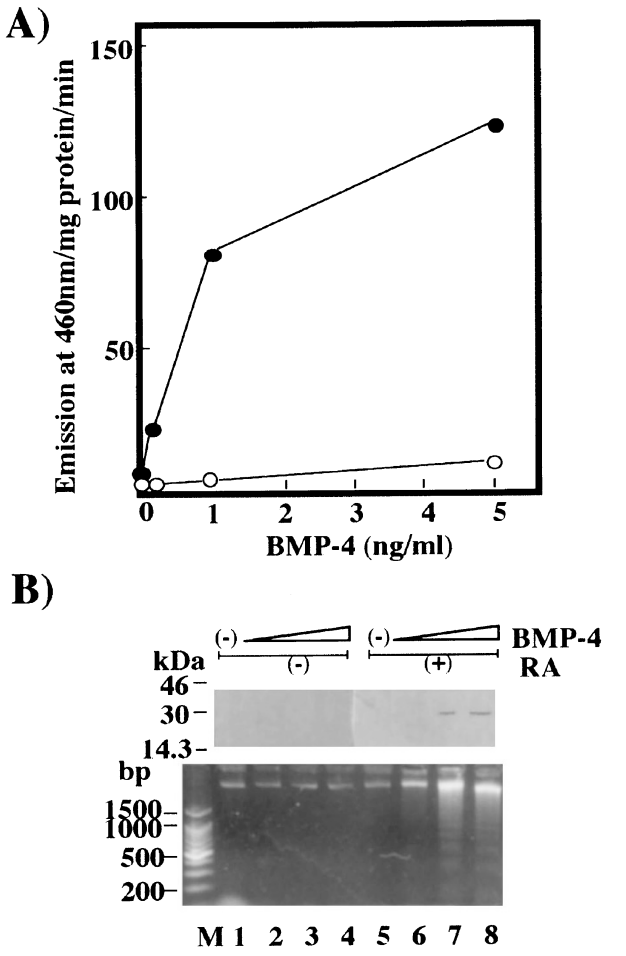

Figure 6 BMP-4 concentration required to induce the synergistic effects on the activation of caspase-9, Ac-DEVD-MCA cleavage activity and DNA fragmentation of P19 EC cells in the cooperation with RA. (**A**) Dose dependent effect of BMP-4 on Ac-DEVD-MCA cleavage activity of P19 EC cells treated with (closed circles) and without (open circles) RA (1000 nM). (**B**) Dose dependent effect of BMP-4 on the processing of caspase-9 (upper panel) and DNA fragmentation (lower panel). Lane 1 and 5; without BMP-4, lane 2 and 6; with BMP-4 at 0.1 ng/ml, lane 3 and 7; with BMP-4 at 1 ng/ml, lane 4 and 8; with BMP-4 at 5 ng/ml. Lane 1–4; without RA and lane 5–8; with RA (1000 nM). M; size marker of DNA

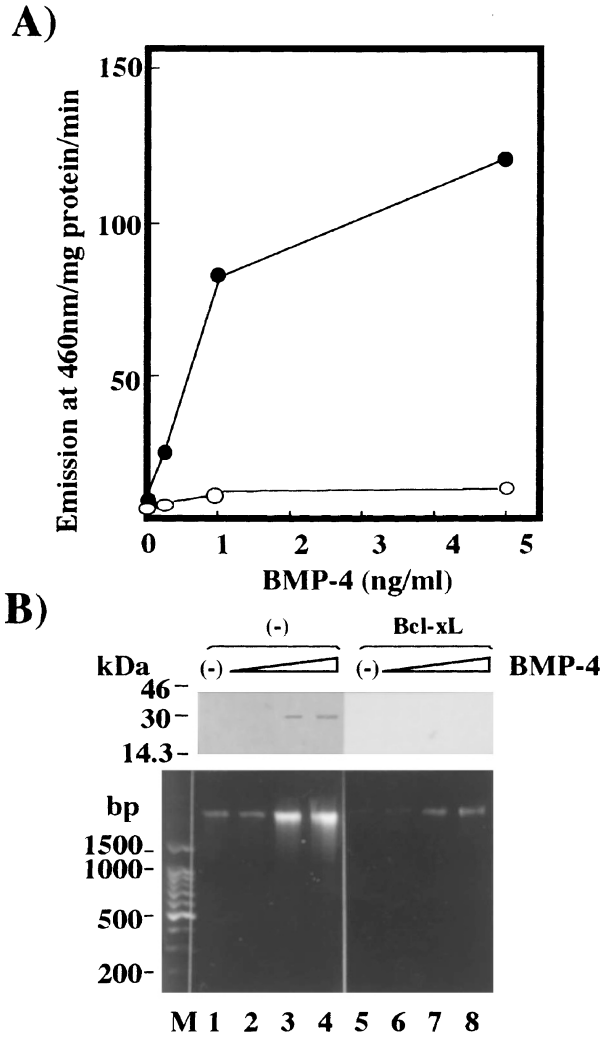

Figure 7 Inhibitory effects of Bcl-xL on the activation of caspase-9 and Ac-DEVD-MCA cleavage activity induced by RA and BMP-4 together. (**A**) Ac-DEVD-MCA cleavage activity in the RA/BMP-4-treated Bcl-xL-transformed and -nontransformed cells. Closed circles; nontransformed P19 EC cells; open circles, Bcl-xL-transformed cells. (**B**) The processing of caspase-9 (upper panel) and DNA fragmentation (lower panel) in the P19 EC cells simultaneously treated with RA (1000 nM) and BMP-4 at different concentration. Lane 1–4, P19 EC cells; lane 5–8, Bcl-xL-transformed cells. Lane 1 and 5, RA-treated cells; lane 2 and 6, RA /BMP-4 (0.1 ng/ml)-treated cells; lane 3 and 7; RA/BMP-4 (1 ng/ml)-treated cells; lane 4 and 8; RA/BMP-4 (5 ng/ml)-treated cells. M; size marker of DNA

the possibility that small amount of caspase-9 was activated by undetectable amount of released cytochrome c.

Down-regulation of XIAP in the RA and BMP-4-treated P19 EC cells

Neuronal apoptosis inhibitor protein (NIAP), a homologue of XIAP, which is a candidate gene for type I spinal muscular atrophy (SMA), has an anti-apoptotic effect on apoptosis induced by a variety of signals, and it contributes to the SMA phenotype when deficient or absent.[25] Very recently, XIAP has been shown to participate in the BMP signaling pathway as a positive regulator linking the BMP receptor and TAK1 and its activator, TAB.[27] Activation of the BMP receptor by ligand binding elicits signals that induce not only ventralization but also apoptosis in *Xenopus* embryos through TAK1 activation.[24] Overexpression of XIAP inhibits TAK1-induced apoptosis and promotes TAK1-induced ventralization,[27] suggesting that XIAP is a key molecule regulating the apoptosis signals in the BMP receptor-TAK1 pathway. The level of XIAP may determine BMP-4-receptor-signal stimulation of the apoptotic pathway and the other signal pathways. The prominent decrease in the level of XIAP in the cytosol of the RA/BMP-4-treated apoptotic cells (Figure 8) may explain their synergistic effect on the activation of caspase-9. A small amount of caspase-9 activated by RA via undetectable levels of released cytochrome c or an unknown apoptotic pathway may induce the processing of a large amount of caspase-9 in

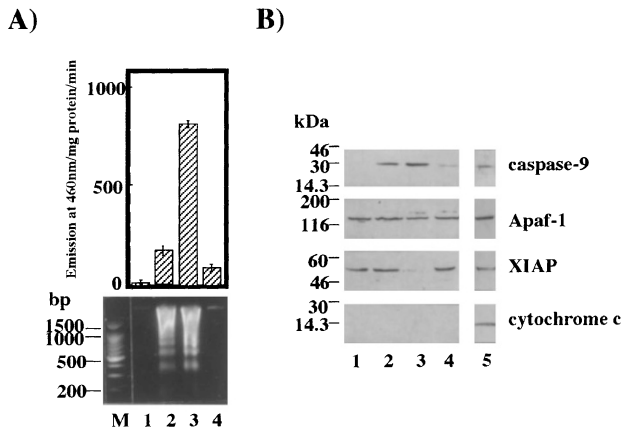

Figure 8 The level of XIAP and cytochrome c in the apoptotic cells of the RA/BMP-4-treated cells. (**A**) DNA fragmentation and Ac-DEVD-MCA cleavage activity in the cytosol fraction of the non-adherent cells and adherent cells of the RA and BMP-4-treated cells. Lane 1; untreated P19 EC cells, lane 2–4; total cells, non-adherent cells and adherent cells of the RA (1000 nM)/BMP-4 (1 ng/ml)-treated P19 EC cells, respectively. (**B**) The level of processed fragment of caspase-9, Apaf-1, XIAP and cytochrome c in the cytosol fraction of the apoptotic cells of the RA and BMP-4-treated cells. Lane 1; untreated P19 EC cells, lane 2–4; total cells, non-adherent cells and adherent cells of the RA/BMP-4-treated P19 EC cells, respectively, lane 5; total cells of staurosporine (1 μM)-treated P19 EC cells

cells, where XIAP is down-regulated by BMP-4 signals. Thus, activation of caspase-9 may be regulated by a balance between apoptotic trigger signal molecules such as cytochrome c or unknown factors and protective factor molecules such as XIAP and members of the NIAP or Bcl-2 family.

Molecular mechanism of RA and BMP-4 inducing synergistic effect on the activation of caspase-9

The reason why BMP-4 alone cannot induce apoptosis and always requires RA synergistically is unclear. One possible explanation is that RA makes P19 EC cells competent to respond to BMP-4 signals or vice versa. BMP-4 receptors type I and type II were upregulated 2-fold in RA-treated cells (data not shown), suggesting that the upregulation of BMP-4 signals induces the synergistic effect of RA and BMP-4 on activation of caspase-9 and apoptosis.

Msx-2, homeobox-containing gene, is one of the possible downstream targets of BMP-4-induced apoptosis signals. Msx-2 is expressed in the same regions in which BMP-4 induces cell death. Disruption of BMP-2 and -4 signaling by a dominant-negative BMP type I receptor prevents interdigital apoptosis and decreases of the Msx-2 expression.[4] Constitutive ectopic expression of Msx-2 in P19 EC cells markedly increases apoptosis induced upon aggregation.[36] However, Msx-2 is not significantly upregulated by RA/BMP-4-treatment (Momoi, unpublished data).

Finally, we do not exclude the possibility that SMADs, the central effector molecules for BMP receptor signals,[37] are involved in RA/BMP-4-induced apoptosis of P19 EC cells, since a dominant-negative mutation of SMAD inhibits processing of caspase-9 induced RA/BMP-4-treatment (Momoi, unpublished data). A further study of the molecular mechanism by which the combination of BMP-4 and RA activates caspase-9 and reduces the level of XAIP in the cytosol is currently underway.

Materials and Methods

Cell culture

P19 EC cells, originally established by McBurney *et al*,[6] were kindly donated by Dr Hamada (Osaka University, Osaka, Japan). All experiments were performed with cells cultured as monolayer with RA (Sigma, St. Louis, MO, USA) and/or BMP-4 (R&D Systems Inc., Minneapolis, MN, USA) unless otherwise noted. Non-adherent RA/BMP-4-treated cells were obtained by collecting the culture medium containing non-adherent cells. Adherent RA/BMP-4-treated cells were obtained by removing the non-adherent cells from the dishes by washing three times with phosphate-buffered saline (PBS).

Ac-DEVD-MCA cleavage activity

P19 EC cells were incubated with RA and/or BMP-4 for various periods. Ac-DEVD-MCA cleavage activity was measured as described previously.[38] After incubation, cells were washed twice with PBS, and the cell pellets were lysed in PBS containing 0.2% Triton X-100 on ice for 10 min. Cells were then centrifuged at 10 000 $\times$ *g* for 5 min. To measure caspase-3-like activity the cell extracts (50 μg protein) were incubated with 10 μM Ac-DEVD-MCA (Peptide Institute, Osaka, Japan), a synthetic peptide of the cleavage site of poly-ADP ribose polymerase, in incubation buffer (50 mM Tris-HCl pH 7.5, 1 mM dithiothreitol) at 37°C for 20 min. The reactions were stopped by the addition of 10% sodium dodecyl sulfate (SDS). Fluorescence intensity was measured at 380 nm for excitation and at 460 nm for emission.

Isolation of fragmented DNA

DNA isolation was performed according to Prigent *et al*,[39] with some modification. Cell pellets were lysed with 400 μl of lysis buffer (20 mM Tris-HCl, pH 7.4, 20 mM EDTA and 1% Triton X-100). Lysis was allowed to proceed on ice for 10 min, and the mixture was centrifuged for 10 min at 10 000 $\times$ *g*. The supernatant was digested with 50 μg/ml RNase A at 37°C for 1 h. This was followed by proteinase K (50 μg/ml) digestion at 37°C for 1 h. After phenol-chloroform extraction, the DNA was precipitated by adding 2.5 vol of 100% ethanol and CH_3COONa (0.3 M final concentration). DNAs were separated on 1.8% NuSieve agarose (FMC Bioproducts, Rockland, ME, USA) gels.

Transfection of caspase-9

A full-length mouse caspase-9 cDNA was obtained from a cDNA library of RA-treated P19 EC cells (Stratagene, La Jolla, CA, USA). The cDNA was inserted into the *EcoR*I site of the pcDEF3 expression vector and transfected into P19 EC cells by the calcium-phosphate method.[40] Eight hours after transfection in the presence or absence of Ac-DEVD-CHO (10 μM), the transfected P19 EC cells were washed with cell culture medium and cultured again in the presence or absence of Ac-DEVD-CHO (10 μM) for the indicated period.

P19 EC cells stably overexpressing Bcl-xL were obtained by transfection with pMKITNeo-bcl-xL and selection with G418 as described previously.[41]

Immunoblot analysis

Cells were lysed in PBS containing 0.2% Triton X-100. After centrifugation at 10 000 × *g* for 10 min, the cell extracts (50 μg protein) were subjected to SDS-acrylamide gel (12%) electrophoresis. After transfer to nitrocellulose membranes, immunoreactivity was detected with anti-caspase-9 (I16), anti-XIAP, anti-Apaf-1 (MBL, Nagoya, Japan), and anti-cytochrome c (clone 7H8.2C2, Pharmingen, San Diego, CA, USA) antibodies and alkaline phosphatase-conjugated goat anti-mouse or rabbit immunoglobulin (Promega, Madison, WI, USA).

Assay for cytochrome c in the cytosol

Cytosolic levels of cytochrome c were examined according to the method of Rosse *et al*.[42] P19 EC cells were treated with RA (1000 nM) and/or BMP-4 (1 ng/ml) for 24 h or staurosporine (1 μM) for 5 h and then cells were collected by centrifugation. Cells pellets were homogenized in 200 μl extraction buffer (10 mM HEPES buffer, pH 7.5, 0.25 M sucrose, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride), and the cytosol and membrane fractions including mitochondria were separated by centrifugation at 14 000 × *g* for 15 min at 4°C. Levels of cytochrome c were examined by immunoblot analysis as described above.

Immunocytochemical staining of activated caspase-3

RA and/or BMP-4-treated P19 EC cells were fixed with 2% paraformaldehyde for 15 min. They were then incubated with anti-p20/17 antibody, an antiserum specific against activated caspase-3, as described previously.[22,43] Immunoreactivity was detected by peroxidase-conjugated avidin-biotin kit (Vectastain ABC kit, Vector Labs, Burlingame, CA, USA) and diaminobenzidine (DAB) (Sigma).

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