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Regulation of Bim in Glucocorticoid-Mediated Osteoblast Apoptosis

B. ESPINA[*], **M. LIANG**, **R.G.G. RUSSELL**, and **P.A. HULLEY**
The Botnar Research Centre, Oxford Institute of Musculoskeletal Sciences, Nuffield Department of Orthopaedic Surgery, Oxford, UK

Abstract

Osteoblasts undergo apoptosis both in vitro and in vivo in response to high dose glucocorticoid (GC) treatment. However, the molecular mechanisms remain elusive, hindering the prevention and treatment of this side-effect. Apoptosis was induced by dexamethasone (Dex) in murine MBA-15.4 osteoblasts within 24–48 h of treatment. We found dose- and time-dependent upregulation of Bim protein, a pro-apoptotic Bcl-2 family member, with highest levels at 24–48 h for 1 μM Dex. This was also observed in primary human bone marrow stromal cells. Bim is subjected to stringent transcriptional and post-translational regulation in osteoblasts. Bim mRNA was upregulated in response to 1 μM Dex; both cycloheximide and the GC receptor antagonist, RU486, prevented Dex-induction of Bim protein, indicating transcriptional regulation involving the GC receptor. The proteasome inhibitor, MG132, potently increased Bim protein levels. Bim was also upregulated in osteoblasts undergoing apoptosis in response to serum deprivation and matrix detachment. Gene silencing experiments show that short interference RNA (siRNA) specific for Bim or the downstream effector Bax both reduced apoptosis induced by Dex in osteoblastic cells. These findings suggest that Bim is a novel regulator of osteoblast apoptosis and may be a therapeutic target.

Glucocorticoid (GC) induced osteoporosis is a major cause of bone loss and fractures. GCs are widely used as anti-inflammatory and immuno-suppressive drugs in the treatment of asthma, collagen-vascular disease, inflammatory bowel disease, granulomatous and skin diseases. The mechanisms by which GCs cause osteoporosis are multiple but include direct and indirect effects on osteoblasts (OB). GCs profoundly repress osteoblast production and promote apoptosis (Weinstein et al., 1998). Supra-physiological doses of GCs dramatically decrease OB number and bone formation rate and this may be explained in part by an increase in OB apoptosis which has been reported in vivo and in vitro (Weinstein et al., 1998; Gohel et al., 1999; Eberhardt et al., 2001).

The cellular effects of GCs are primarily mediated by the glucocorticoid receptor (GR). The GR is widely expressed in most cell types, including bone (Abu et al., 2000). In the absence of ligand the GR is localized in the cytoplasm and upon ligand binding the receptor translocates into the nucleus where it uses a variety of effector mechanisms. These include binding as a homodimer to positive or negative regulatory elements in promoter regions of GC-responsive genes (GRE), resulting in modulated gene transcription (Schacke et al., 2002) and negative interference with other transcription factors such as AP1 and NFkappaB (De Bosscher et al., 2003).

[*]Correspondence to: B. Espina, The Botnar Research Centre, University of Oxford Institute of Musculoskeletal Sciences, Nuffield Department of Orthopaedic Surgery, Oxford OX3 7LD, UK. mbespina@humv.es.

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Several molecular mechanisms appear to be involved in GC-induced apoptosis in susceptible cells such as lymphocytes, osteocytes, and osteoblasts. The activated GR initiates a transcriptional program involving calcium mobilization, cytochrome c release and caspase activation leading to cell death (Cifone et al., 1999; Marchetti et al., 2003), but the critical signalling intermediates in GC-induced apoptosis remain largely undefined. Increasing evidence implicates Bcl-2 Interacting Mediator (Bim of cell death), one of the members of the BH3-only proteins, as a critical trigger of apoptosis in many cell types (O'Connor et al., 1998). Bim belongs to the Bcl-2 family of cytoplasmic proteins, central regulators of apoptosis in the mitochondrial pathway. All members have at least one of four conserved motifs known as Bcl-2 homology regions (BH1–BH4). Bcl-2 and other close relatives inhibit apoptosis, whereas relatives such as Bax or Bim promote cell death.

Bim, like other BH3-only proteins, is subject to stringent control at both the transcriptional and the post-translational level (Strasser et al., 2000). De novo expression of Bim is a major mechanism of regulation. In addition, Bim is regulated by sequestration to cytoskeletal structures inside cells (Strasser et al., 2000) and by phosphorylation leading to ubiquitinylation and consequent proteasomal degradation (Ley et al., 2003). Three major isoforms have been described, BimEL (extra long), BimL (long) and BimS (short), which vary in potency and appear to be regulated in different ways (O'Connor et al., 1998; Puthalakath et al., 1999). Bim acts at the mitochondria either directly, inducing Bax and Bak oligomerization, or indirectly, through decoy interactions with the anti-apoptotic molecules (such as Bcl-2 and Bcl-xL) (Kim et al., 2006).

Bim has been shown in a variety of cell types to be sufficient for induction of apoptosis in vitro. Bim −/− mice revealed non-redundant roles for this protein since at least in the haematopoietic compartment Bim is a key trigger of apoptosis. Thymocytes, lymphocytes and granulocytes in Bim −/− mice are resistant to cytokine withdrawal and chemotherapeutic drugs such as taxol (Bouillet et al., 1999). Recently Bim has been identified as a GC-induced gene (Wang et al., 2003), with upregulation at both the mRNA and the protein level reported in multiple examples of GC-induced apoptosis (Bouillet et al., 1999; Han et al., 2001; Wang et al., 2003). Another BH3-only protein, PUMA (previously bbc3), has also been reported to be upregulated at the gene level by GCs (Han et al., 2001). Deletion of Bim strongly protects thymocytes against GC-induced apoptosis (Erlacher et al., 2005), supporting the role of Bim as a death mediator in GC induced apoptosis. In bone, Bim is essentially required for M-CSF withdrawal-induced apoptosis in osteoclasts (Akiyama et al., 2003). However Akiyama et al. (2003) reported that Bim is not expressed in other skeletal cells such as osteoblasts and chondrocytes during development or in the healthy adult. Whether there is a relevant role for Bim in these cells is still unknown.

In the current report we have identified Bim as a novel pro-apoptotic marker in both human and MBA 15.4 mouse osteoblastic cells and describe its regulation in a newly developed in vitro model of GC-induced apoptosis. The MBA 15.4 osteoblastic cell line has proved to be a useful osteoblastic differentiation model and is able to form bone in vitro and in vivo (Fried et al., 1993). MBA 15.4 cells are also sensitive to high-dose GC and dexamethasone (Dex) strongly represses proliferation in these cells (Fried et al., 1993; Hulley et al., 1998).

Materials and Methods

Reagents

Dexamethasone, cycloheximide, actinomycin D and RU486 (mifepristone) were obtained from Sigma (Sigma–Aldrich Ltd, Poole, UK). The proteasome inhibitor MG132 was obtained from Calbiochem (San Diego, CA). Cycloheximide was used at final concentrations of 0.5 μg/ml (protein synthesis inhibition) and 40 μg/ml (positive control for

apoptosis). Control in all experiments is vehicle (DMSO and/or ethanol) unless otherwise indicated.

Cell culture and isolation of human bone marrow stromal cells (hBMSC)

Tissue culture media, media supplements and antibiotics were purchased from Cambrex (Wokingham, UK). All media were supplemented with 10% heat-inactivated fetal calf serum (FCS) (Biowest, Ringmer, UK).The murine pre-osteoblastic cell line MBA 15.4 (kindly provided by Dafna Benayahu, Tel Aviv University, Israel) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10%FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

For isolation of hBMSC, bone marrow was obtained from the upper femur of patients undergoing total hip replacement surgery for primary osteoarthritis with informed consent and the approval of the hospital ethics committee. Samples were cut into small pieces and suspended in supplemented media. Samples were then vortexed vigorously, poured through a 70 μm cell strainer and centrifuged. Stromal cells were selected by their ability to adhere to plastic and used for experiments between passages one and three.

For the *anoikis* model sterile 1% agarose was heated and poured into cell culture plates. Cell monolayers were trypsinised and transferred to the agarose coated plates for the indicated time intervals.

Apoptosis assays

Nick translation (NT)—Cells demonstrating DNA breaks were investigated in samples fixed in 4% formaldehyde, resuspended in 100% ethanol and stored at −20°C overnight. Cells were washed and incubated with a NT mixture which consisted of 2.5 mM $MgCl_2$, 50 mM Tris, 10 μg/ml bovine serum albumin (BSA), 2.5 μl DNA polymerase, 10 mM β-mercaptoethanol, 1μM of each dATP, dCTP, dGTP and 1 μM biotin-16-dUTP and incubated for 4 h at room temperature. Cells were then washed and resuspended in the staining buffer (consisting of 0.1% Triton X-100, 4× SSC, 5% non-fat dry milk and 2.5 μg/ml avidin conjugated to fluorescein isothiocyanate (FITC)) (Gorczyca et al., 1993). Cells were subjected to flow cytometry (FACScan and BD CellQuest Pro software v4.0.2, Becton Dickinson UK Ltd, Oxford, UK) and analyzed for forward and side light scatter, as well as FITC fluorescence. For each measurement a minimum of 5,000 cells was analyzed.

Caspase 3 and 7 activity assay—Apo-ONE Caspase-3/7 reagent (Promega, Madison, WI) was utilized to measure effector caspases 3/7 activity.

Acridine orange (AO) staining—Cells cultured on glass coverslips under different conditions were fixed in Walpole's buffer ph 4.2 (0.2 M HCl, 0.3 M sodium acetate) followed by AO staining for 20 min at room temperature (Kogianni et al., 2004).Cells which showed apoptotic morphology (membrane blebbing, shrinkage or nuclear fragmentation) and normal cells were counted.

Western blot analysis

Bim and anti-cleaved caspase 3 polyclonal antibodies were purchased from Calbiochem. Pro-apoptosis Bcl-2 family polyclonal antibodies (Bax and Puma) were obtained from Cell Signalling Technologies (Beverly, MA). Pan Bak antibody was obtained from Upstate (Dundee, UK). Anti-Bcl-2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GAPDH antibody was obtained from Abcam (Cambridge, UK). Total cell proteins were extracted by sonication in a buffer containing: 1 mM EDTA, 1 mM PMSF,

1% NP-40 and 0.1% SDS and electrophoresed on SDS–PAGE gels. Western blots of gels were analyzed for the proteins of interest using rabbit polyclonal antibodies and alkaline phosphatase color reaction. Immune complexes were visualized using the enhanced chemiluminiscence detection system and bands were measured by densitometric analysis using ImageJ version 1.32j software. The intensity of each band was normalized to that of the GAPDH protein band.

Real time quantitative PCR

Total RNA was isolated from cultured cells using RNeasy Mini kits (Qiagen, GmbH, Hilden, Germany), according to the manufacturer's protocol. RNase-Free DNase Set (Qiagen GmbH) was used to remove genomic DNA contamination. 0.3 μg total RNA in a 20 μl reaction volume was reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen, Paisley, UK) in the presence of oligo-dT primer (Invitrogen). Primers were from MWG Biotech (London, UK). Mouse *BIM* forward primer was 5′-CGACAGTCTCAGGAGGAACC-3′ and reverse primer was 5′-CCTTCTCCATACCAGACGGA-3′. Mouse *GAPDH* forward primer was 5′-GGTCATCCCAGAGCTGAACG-3′ and reverse primer was 5′-TTGCTGTTGAAGTCGCAGGA-3′. Real time quantitative PCR reactions were performed using a Corbett Rotor-Gene 3000 using QuantiTect SYBR Green PCR kit (Qiagen, GmbH). Comparative Quantitation analysis (Warton et al., 2004) was used to compare the gene expression of Bim in treated samples relative to control cells and normalized to the housekeeping gene GAPDH (Rotor-Gene version 6.0.1 software). Data was obtained from three independent experimental repeats.

Transfection with Bim and Bax small interfering RNA (siRNA)

Cells were pre-plated in six-well plates, in antibiotic-free medium containing 10% FCS, 24 h before transfection and reached 40% confluence at the time of transfection. Optimal concentrations of siRNA were determined in preliminary experiments as previously reported (Liang et al., 2007). It was found that Bim could be knocked down within 24 h while Bax was a more stable protein and required 48 h post-transfection. Thirty nanomolars Bim (siBim) and 30 nM Bax (siBax) siRNA as well as 30 nM scrambled control (siControl) siRNA (all from Ambion, Huntingdon-Cambridgeshire, UK) were introduced into the cells using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen), according to the manufacturer's recommendations. The sense strand sequences were: *BIM* 5′-GGUGGACAAUUGCAGCCUGtt-3′; *BAX* 5′-GGCCCUGUGCACUAAAGUGtt-3′. For the last 18 h of transfection the cells were deprived of serum. Twenty four hours after transfection with Bim siRNA or 48 h after transfection with Bax siRNA, protein levels of Bim and Bax were determined in parallel to confirm specific gene knockdown using Western blotting. Bak and/or GAPDH was used as internal control/s. An in situ cell death detection kit (Roche Applied Science, Penzberg, Germany) was used to perform the TUNEL reaction to identify apoptotic cells with fragmented DNA, according to the manufacturer's instructions. DAPI staining was used to visualize all nuclei. The cells were observed and photographed using Olympus BX40 microscope and Olympus DP70 camera. TUNEL- and DAPI-positive cells were counted using cell F program (Olympus, Soft Imaging System GmbH, Münster, Germany) and at least 15 fields were taken from each slide. Data was obtained from four independent experimental repeats

Statistical analysis

Data were analyzed by parametric one-way analysis of variance (ANOVA). Statistical analysis was carried out using SPSS version 12.0 (2006 SPSS, Inc., Chicago, IL) with P values less than 0.05 considered significant. Dunnett test was used as a post-hoc test. t-test for independent samples was used when comparing only two groups of data.

Results

Dexamethasone induces apoptosis in the mouse osteoblastic cell line MBA 15.4

We firstly determined whether the murine osteoblastic cell line MBA 15.4 undergoes cell death when treated with the synthetic GC, dexamethasone (Dex), in vitro. Osteoblastic cells were exposed to a wide range of concentrations of Dex (1 nM, 10 nM, 100 nM, 1 μM, 5 μM, and 10 μM) for different times (12 h up to 72 h) and cell death was measured, initially using Trypan blue staining. Increasing rates of cell death (~3-fold) were found for Dex at doses 1 and 5 μM for 24–48 h exposure times (data not shown), in agreement with studies in primary osteoblasts and other osteoblastic cell lines. Apoptotic cell death was further assessed using nick translation assay (NT). Dex treatment induced a threefold increase in apoptotic osteoblast numbers after 24 h (14.4% ± 5.2 compared to 5.6% ± 3.6 in control), reaching fourfold when increasing the dose (5 μM) and the exposure to Dex up to 48 h (24% ± 7.6 compared to 5.6% ± 3.6 in control) (Fig. 1A). Cycloheximide at the cytotoxic dose of 40 μg/ml was used as a positive control for apoptosis induction.

Dex treatment activates caspases 3/7 transiently, and this was first detected at 36 h just before the maximum rates of apoptosis at 48 h (Fig. 1B). Although caspase 3 activation normally precedes DNA fragmentation, DNA fragmentation was first detected within 24 h of Dex treatment. This is most likely to due to the stable, high sensitivity nature of DNA detection and the transient and insensitive assay for caspase 3 activity. Active caspase 3 has recently been reported to be proteasomally degraded which is likely to limit the range of detectable signal in a cell population to the peak apoptotic period (Suzuki et al., 2001). Serum deprivation was used as a good positive control for apoptosis induction since more than 30% of the cells die within 24 h, with concurrent activation of caspase 3 (Figs. 1B and 3B). When visualized by acridine orange staining apoptotic osteoblasts showed the typical morphology (Fig. 1C).

Dexamethasone induces an increase in Bim protein and mRNA

Bim protein expression levels were investigated in MBA15.4 osteoblasts. Whilst Bim levels were barely detectable in the control, Dex increased the levels at the indicated doses and times. The most abundant isoform, BimEL, was quantified. Protein levels peaked at 24 h after treatment with Dex 1 μM (sixfold increase vs. solvent control), remained elevated up to 48 h and decreased at 72 h (Fig. 2A). However, we found that the peak response time varied between 24 and 48 h. This osteoblast cell line underwent a threefold increase in apoptosis after 24–48 h treatment with Dex 1 μM (nick translation assay). Moreover, different doses of Dex were tested at 48 h and Bim expression increased from Dex 1 nM to a peak at Dex 1 μM, and then decreased at Dex 10 μM (Fig. 2B). At the dose of 10 μM necrotic features were observed when the cells were stained with Trypan blue. Data from real-time quantitative PCR also demonstrated that there was an increase in Bim mRNA following treatment with Dex 1 μM. This increase was time-dependent, reaching significance at 16 h and remaining elevated until at least 24 h (~2-fold compared to control; Fig. 2C).

The protein expression of PUMA was also examined. Cells were exposed to Dex for 48 h, which was one of the time points with highest rate of apoptosis, at doses 1 nM up to 10 μM. The pro-apoptotic protein PUMA was highly expressed in OB in contrast to Bim. However, PUMA protein levels did not increase after exposure to Dex at the times and doses investigated (Fig. 2A,B). We further investigated the expression of Bcl-2 and Bax, mitochondrial proteins which signal downstream of Bim. Protein expression of Bcl-2 and Bax was not regulated after treatment of MBA 15.4 cells with Dex at any dose or time tested (Fig. 2A,B).

We then confirmed Dex induction of Bim protein in hBMSC. Undifferentiated human bone stromal cells were treated with 1 μM Dex for different times. Basal levels of Bim protein were barely detectable when approximately 15 μg of protein were loaded. Bim protein levels increased up to four fold after 36 h of treatment with Dex (Fig. 3A).

Bim protein is also regulated in osteoblastic cells by growth factor deprivation and by detachment

We have previously shown that MBA 15.4 cells became apoptotic after serum withdrawal (De Wet et al., 2003; Espina et al., 2005). In order to examine Bim protein expression following growth factor depletion, MBA 15.4 osteoblastic cells were cultured until subconfluency in 10% FCS and then changed to 0% FCS for 4 to 10 h. BimEL protein levels increased by 7-fold after 4 h of FCS withdrawal and up to 16-fold after 10 h. Cleaved (active) caspase 3 protein levels were not apparent until 8 h following serum removal and increased to 45-fold after 10 h (Fig. 3B).

Bim has also been proposed as a key sensor of integrin activity in epithelial cells (Reginato et al., 2003). We next examined the expression of Bim after detachment of MBA15.4 osteoblasts by plating the cells on agarose. BimEL isoform protein levels increased strongly following detachment. The maximum levels were reached within 24–48 h of detachment and remained high for at least 72 h (nine- and fivefold, respectively; Fig. 3C). During this time massive osteoblast cell death occurred (more than 40% after 24 h) when measured by the Trypan blue assay (data not shown).

Interaction with the glucocorticoid receptor and de novo synthesis of protein are both required for Bim expression following Dex treatment

Interaction of the GC with the GR and translocation to the nucleus is needed to induce apoptosis (Cifone et al., 1999; Katychev et al., 2003). In bone, the GC receptor antagonist RU486 abrogated apoptosis induction by Dex in osteocytes (Gu et al., 2005). We therefore examined whether RU486 could block Bim expression in osteoblastic cells. Co-treatment with Dex (100 nM) and a ten-fold excess of RU486 markedly reduced Bim expression (Fig. 4A).

To determine whether transcription and/or new protein synthesis were necessary for Dex-induced Bim expression we treated MBA 15.4 cells with Dex 1 μM in the presence or in the absence of non-toxic doses of the transcription inhibitor, actinomycin D, and/or the protein synthesis inhibitor, cycloheximide, for 24 h. Actinomycin D (10 nM) alone increased BimEL and failed to prevent Bim expression in the presence of Dex. However, BimEL induction by Dex was significantly reduced when cells were treated either with cycloheximide (0.5 μg/ml) or with both cycloheximide and actinomycin D (Fig. 4B).

Bim protein levels are regulated by the 26S proteasome in osteoblasts

To determine if Bim is degraded via the proteasome in osteoblasts, MBA 15.4 cells were cultured in the presence of 10% FCS and the proteasome inhibitor MG132. Bim protein levels strongly increased, up to threefold (3 ± 1.3), after treatment with MG132 for 24 h (Fig. 4C). We further investigated the expression of the pro-apoptotic protein, Bax, which has been shown to be regulated by the proteasome in certain cell types (Li and Dou, 2000). Bax protein levels remained unchanged after treatment with the proteasome inhibitor in MBA 15.4 cells (Fig. 4C).

Silencing of Bim or the downstream effector Bax results in a significant reduction of dexamethasone-induced apoptosis in MBA 15.4 osteoblastic cells

Although it is clear that Bim is strongly upregulated at the mRNA and the protein level in MBA 15.4 osteoblasts in response to dexamethasone treatment it is unclear whether Bim is directly implicated in the induction of apoptosis by glucocorticoids. In order to address this issue we performed gene silencing experiments using Bim-specific siRNA. For Bim siRNA optimization and since constitutive levels of Bim are hardly detectable in these cells under control conditions, we cultured the cells in the absence of serum to strongly upregulate Bim. As shown in Figure 5A (left part), Bim protein levels were strongly knocked-down by Bim siRNA after 24 h whereas the scrambled siControl had no effect. We further confirmed the specificity of Bim siRNA by performing Western blots for the downstream effectors of Bim, Bax and Bak in the same samples. The total levels of these proteins were not affected by siBim, nor was the unrelated housekeeper, GAPDH (Fig. 5A, left part). We then investigated whether the reduction in Bim levels by siBim could inhibit apoptosis induced by Dex in these cells. Basal rate of apoptosis by TUNEL was less than 1%, with or without siControl (data not shown). Transfection with Bim-specific siRNA clearly caused a strong reduction in Dex-induced apoptosis in osteoblastic cells (reducing from 9% in the presence of siControl to 3% in the presence of siBim) (Fig. 5A, right part).

We further investigated whether the gene silencing of the downstream effector of Bim, Bax, could also inhibit Dex-induced apoptosis in MBA 15.4 cells. Bax levels were high in untreated cells and were unaffected by either serum deprivation (Fig. 5B, left part) or Dex (see Fig. 2A,B). Bax protein was strongly and selectively knocked down by siBax 48 h after transfection (Fig. 5B, left part), while siControl had no effect. Again we investigated whether the reduction in Bax levels by siBax could inhibit apoptosis induced by Dex in these cells. Bax siRNA significantly inhibited Dex-induced apoptosis in MBA 15.4 osteoblasts (reducing from 8% in the presence of siControl to 2% in the presence of siBax) (Fig. 5B, right part).

Discussion

High dose glucocorticoid treatment induces osteoporosis partly via increasing osteoblast and osteocyte apoptosis and this seems to be mediated by regulation of gene expression, although the target genes have not been identified (Weinstein et al., 1998; Galon et al., 2002; Wang et al., 2003; Zalavras et al., 2003). The pro-apoptotic protein Bim has recently been shown to be induced following Dex treatment in haemopoietic cells undergoing GC-induced apoptosis and is capable of directly initiating the apoptosis cascade (Wang et al., 2003). Although Bim has been reported as not expressed in osteoblasts (Akiyama et al., 2003), in this study we show that Bim is induced by Dex in a dose- and time-dependent manner in mouse osteoblastic cells in vitro. Moreover, increased levels of BimEL correlate in time and dose with significantly higher rates of apoptosis in these cells. We have also confirmed a role for Bim in this model since knockdown of Bim protein by RNA interference strongly reduced Dex-induced apoptosis in osteoblastic cells.

Several groups have reported increased induction of apoptosis by glucocorticoids in vitro, in either murine or rat primary OB or osteoblastic cell lines (Gohel et al., 1999; Plotkin et al., 1999; Yamamoto et al., 2002; Ahuja et al., 2003). We report here that in MBA15.4 osteoblastic cells Dex-induced apoptosis peaks between 24 and 48 h of treatment. Other studies in osteoblasts and osteocytes in vitro also observed apoptosis after longer exposure times to GCs (Gohel et al., 1999; Gu et al., 2005). In keeping with previous literature we have shown that in OB cells, which have a low basal apoptotic index (number of apoptotic cells relative to healthy cells), Dex only induces a three- to fourfold increase in apoptosis. However, apoptosis following Dex treatment in our model is characterized by DNA

fragmentation and typical morphological features and was dose and time dependent. MBA-15.4 osteoblasts also displayed caspase 3 and 7 activation, which is a hallmark of apoptosis that has been previously reported in other osteoblastic models (Chua et al., 2003; Kogianni et al., 2004).

We found some variation regarding apoptosis index in response to Dex depending on the apoptosis detection method used (approximately 4% apoptotic cells following Dex treatment detected by acridine orange compared with 15% apoptotic cells by Nick translation). This relatively subtle variation is possibly due to different sensitivities of the apoptosis detection techniques. An alternative explanation is that these techniques measure apoptosis at different stages, with DNA fragmentation preceding overt nuclear breakdown and blebbing. Apoptosis is a transient process with some events, such as phosphatidyl serine exposure on the plasma membrane occurring rapidly and others, such as DNA fragmentation being more stable.

The BH3-only protein Bim is a death mediator and its involvement has been described in multiple examples of trophic factor deprivation such as in osteoclasts (Akiyama et al., 2003), hematopoietic cells (Dijkers et al., 2000) or fibroblastic cells (Ley et al., 2003). We demonstrate here that healthy primary hBMSCs and osteoblastic MBA 15.4 cells express very low levels of Bim protein and only BimEL isoform is detectable. However, stressed OB express BimEL, BimL and sometimes BimS isoforms, although BimEL is the most abundant form and the most easily detectable, as demonstrated in other cell types (Wang et al., 2003).

We have shown that Dex regulates Bim in a dose- and time-dependent manner in mouse osteoblastic cells in vitro but does not regulate the expression of Bcl-2 or Bax protein at the times and doses investigated. These findings are in contrast to previous observations showing downregulation of Bcl-2 and/or upregulation of Bax (reduced Bcl-2/Bax ratio) by Dex treatment (Gohel et al., 1999; Planey et al., 2002). Perhaps these different results could be explained by differences amongst cell models. Over-expression of Bcl-2 has proven to be protective in multiple models of apoptosis including GC-induced apoptosis (Caron-Leslie et al., 1994; Memon et al., 1995). However, recent evidence suggests that the levels of Bim primarily modulate GC-resistance, independently of Bcl-2, in GC-induced apoptosis in lymphocytes (Abrams et al., 2004). In addition, the pro-apoptotic protein, PUMA, is not regulated by Dex in our model in contrast to some reports in non-osteoblastic cell types (Han et al., 2001) suggesting that different cell types may require different BH3-only proteins to activate programmed cell death.

Bim expression increases after growth factor deprivation in osteoblastic MBA 15.4 cells and this is in keeping with other studies (Dijkers et al., 2000; Ley et al., 2003). In our model Bim expression occurs prior to activation of caspase 3 and suggests that BimEL expression is an upstream event during apoptosis of osteoblastic cells. This rapid (4 h) induction of Bim after serum withdrawal was in contrast to the slower induction of Bim by Dex after 12–24 h. These results suggest that Bim may be differentially regulated in these models, perhaps due to the necessarily indirect transcriptional induction by GC in the absence of a Bim GRE (Wang et al., 2003). We have also investigated the expression of Bim in response to detachment, since it has been described that Bim is involved in *anoikis* in cells that grow in adherent conditions such as epithelial cells (Reginato et al., 2003; Marani et al., 2004). Since Bim is expressed in response to other stimuli in addition to dexamethasone this indicates that Bim is not a specific Dex-induced apoptosis marker but a more general apoptotic marker. However it is required for Dex-induced apoptosis since we demonstrate also that specific knockdown of Bim using RNA interference was strongly protective against Dex-induced apoptosis.

De novo expression of Bim is a major mechanism of apoptotic regulation in response to GC treatment and others insults (Cifone et al., 1999; Ley et al., 2003). Our results suggest that binding to the cytoplasmic GR and translation of new protein are both necessary for induction of Bim expression by Dex, although the mechanism is likely to be indirect and complex since the Bim gene contains no canonical GRE (Wang et al., 2003). Interestingly the transcriptional inhibitor, actinomycin D, either alone or in addition to Dex, caused an increase in Bim protein (~2-fold) that was abrogated when the translational inhibitor, cycloheximide, was added. This may indicate that actinomycin D stabilizes Bim mRNA by inhibiting the synthesis of a protein responsible for Bim mRNA or protein degradation, thus leading to Bim protein accumulation. We have also shown using real time quantitative PCR that there is an increase in Bim mRNA following Dex treatment. These data indicate that Bim protein expression in response to Dex is due to increased Bim transcription.

Recent studies have outlined that the ubiquitin-proteasome pathway mediates the degradation of key apoptotic regulatory proteins (Jesenberger and Jentsch, 2002). We have shown in this report that proteasomal inhibition dramatically increases Bim protein levels in osteoblastic cells indicating that Bim is continuously being degraded by the proteasome to maintain relatively low basal levels. This seems to be a general regulatory mechanism since it has been reported in different cell types (Ley et al., 2003; Luciano et al., 2003).

The results shown here also demonstrate that when Bim is knocked-down by specific silencing RNA, apoptosis induced by dexamethasone is significantly reduced (approximately 60% reduction). This indicates that Bim has a functional role in Dex-induced apoptosis in osteoblastic cells. To our knowledge this the first time this is reported in osteoblastic cells. Previous reports have shown similar results in lymphocytic cells targeting Bim with small interference RNA technology (Abrams et al., 2004; Essafi et al., 2005) or using cells from Bim deficient mice (Bouillet et al., 1999).

The pro-apoptotic function of Bim is thought to require Bax (Kim et al., 2006). The pro-apoptotic Bax and its relative Bak are both multidomain members of the Bcl-2 family of mitochondrial proteins. Notably Bax can form pores (and probably Bak) in the mitochondrial external membrane, resulting in the release of cytochrome *c* into the cytosol, followed by activation of caspase 3 (Donovan and Cotter, 2004). The role of Bax in Dex-induced apoptosis was also investigated here. Bax protein expression was also knocked-down by RNA interference and apoptosis following Dex treatment was clearly reduced (approximately 70% reduction).These results indicate that Bim plays a key role in Dex-induced apoptosis signaling and this is probably mediated through interaction with the downstream effector Bax. Since Bax activation in the mitochondria is well-known to result in sequential activation of caspases 9 and 3 the data provides, in addition, a molecular explanation of the Dex-induced activation of caspase 3.

In summary, contrary to previous reports (Akiyama et al., 2003) Bim is expressed in osteoblastic cells and is co-regulated with apoptosis under conditions of stress such as high-dose glucocorticoid treatment, as well as growth factor deprivation or detachment. In osteoblasts GC-induced upregulation of Bim requires GC:GR interaction and de novo protein synthesis, and as in other cell types, Bim is constantly subjected to proteasomal degradation. We have confirmed a functional correlation between levels of Bim (or the downstream effector Bax) and the degree of apoptosis induced by dexamethasone. We therefore suggest that Bim is a novel regulator of OB apoptosis in response to high-dose glucorticoids and may be a target for therapeutic approaches. Establishment of its physiological roles and how damage signals relay through Bim will facilitate manipulation of pro-survival signaling in vulnerable skeletal cells in the clinical setting.

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Literature Cited

Abrams MT, Robertson NM, Yoon K, Wickstrom E. Inhibition of glucocorticoid-induced apoptosis by targeting the major splice variants of BIM mRNA with small interfering RNA and short hairpin RNA. J Biol Chem. 2004; 279:55809–55817. [PubMed: 15509554]

Abu EO, Horner A, Kusec V, Triffitt JT, Compston JE. The localization of the functional glucocorticoid receptor alpha in human bone. J Clin Endocrinol Metab. 2000; 85:883–889. [PubMed: 10690906]

Ahuja SS, Zhao S, Bellido T, Plotkin LI, Jimenez F, Bonewald LF. CD40 ligand blocks apoptosis induced by tumor necrosis factor alpha, glucocorticoids, and etoposide in osteoblasts and the osteocyte-like cell line murine long bone osteocyte-Y4. Endocrinology. 2003; 144:1761–1769. [PubMed: 12697681]

Akiyama T, Bouillet P, Miyazaki T, Kadono Y, Chikuda H, Chung UI, Fukuda A, Hikita A, Seto H, Okada T, Inaba T, Sanjay A, Baron R, Kawaguchi H, Oda H, Nakamura K, Strasser A, Tanaka S. Regulation of osteoclast apoptosis by ubiquitylation of proapoptotic BH3-only Bcl-2 family member Bim. EMBO J. 2003; 22:6653–6664. [PubMed: 14657036]

Bouillet P, Metcalf D, Huang DC, Tarlinton DM, Kay TW, Kontgen F, Adams JM, Strasser A. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science. 1999; 286:1735–1738. [PubMed: 10576740]

Caron-Leslie LA, Evans RB, Cidlowski JA. Bcl-2 inhibits glucocorticoid-induced apoptosis but only partially blocks calcium ionophore or cycloheximide-regulated apoptosis in S49 cells. FASEB J. 1994; 8:639–645. [PubMed: 8005391]

Chua C, Chua B, Chen Z, Landy C, Hamdy R. Dexamethasone induces caspase activation in murine osteoblastic MC3T3-E1 cells. Biochim Biophys Acta. 2003; 1642:79–85. [PubMed: 12972296]

Cifone MG, Migliorati G, Parroni R, Marchetti C, Millimaggi D, Santoni A, Riccardi C. Dexamethasone-induced thymocyte apoptosis: Apoptotic signal involves the sequential activation of phosphoinositide-specific phospholipase C, acidic sphingomyelinase, and caspases. Blood. 1999; 93:2282–2296. [PubMed: 10090938]

De Bosscher K, Vanden Berghe W, Haegeman G. The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: Molecular mechanisms for gene repression. Endocr Rev. 2003; 24:488–522. [PubMed: 12920152]

De Wet H, Conradie M, Langeveldt CR, Hulley PA. The identification of trancriptional targets associated with GC-induced apoptosis and survival in human and mouse osteoblast cell lines. Eur J Biochem. 2003; 270:PS01–0318.

Dijkers PF, Medema RH, Lammers JW, Koenderman L, Coffer PJ. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. Curr Biol. 2000; 10:1201–1204. [PubMed: 11050388]

Donovan M, Cotter TG. Control of mitochondrial integrity by Bcl-2 family members and caspase-independent cell death. Biochim Biophys Acta. 2004; 1644:133–147. [PubMed: 14996498]

Eberhardt AW, Yeager-Jones A, Blair HC. Regional trabecular bone matrix degeneration and osteocyte death in femora of glucocorticoid-treated rabbits. Endocrinology. 2001; 142:1333–1340. [PubMed: 11181552]

Erlacher M, Michalak EM, Kelly PN, Labi V, Niederegger H, Coultas L, Adams JM, Strasser A, Villunger A. BH3-only proteins Puma and Bim are rate-limiting for gamma-radiation- and glucocorticoid-induced apoptosis of lymphoid cells in vivo. Blood. 2005; 106:4131–4138. [PubMed: 16118324]

Espina B, Conradie R, Russell G, Hulley PA. The BH3-only protein Bim is upregulated in osteoblasts in response to dexamethasone treatment, growth factor deprivation and anoikis. Bone. 2005; 36:S103–S479. [PubMed: 16082734]

Essafi A, Fernandez de Mattos S, Hassen YA, Soeiro I, Mufti GJ, Thomas NS, Medema RH, Lam EW. Direct transcriptional regulation of Bim by FoxO3a mediates STI571-induced apoptosis in Bcr-Abl-expressing cells. Oncogene. 2005; 24:2317–2329. [PubMed: 15688014]

Fried A, Benayahu D, Wientroub S. Marrow stroma-derived osteogenic clonal cell lines: Putative stages in osteoblastic differentiation. J Cell Physiol. 1993; 155:472–482. [PubMed: 8388001]

Galon J, Franchimont D, Hiroi N, Frey G, Boettner A, Ehrhart-Borstein M, O'shea JJ, Chrousos GP, Bornstein SR. Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on inmune cells. FASEB J. 2002; 16:61–71. [PubMed: 11772937]

Gohel A, McCarthy MB, Gronowicz G. Estrogen prevents glucocorticoid-induced apoptosis in osteoblasts in vivo and in vitro. Endocrinology. 1999; 140:5339–5347. [PubMed: 10537165]

Gorczyca W, Melamed MR, Darzynkiewicz Z. Apoptosis of S-phase HL-60 cells induced by DNA topoisomerase inhibitors: Detection of DNA strand breaks by flow cytometry using the in situ nick translation assay. Toxicol Lett. 1993; 67:249–258. [PubMed: 8383887]

Gu G, Hentunen TA, Nars M, Harkonen PL, Vaananen HK. Estrogen protects primary osteocytes against glucocorticoid-induced apoptosis. Apoptosis. 2005; 10:583–595. [PubMed: 15909120]

Han J, Flemington C, Houghton AB, Gu Z, Zambetti GP, Lutz RJ, Zhu L, Chittenden T. Expression of bbc3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals. Proc Natl Acad Sci USA. 2001; 98:11318–11323. [PubMed: 11572983]

Hulley PA, Gordon F, Hough FS. Inhibition of mitogen-activated protein kinase activity and proliferation of an early osteoblast cell line (MBA 15.4) by dexamethasone: Role of protein phosphatases. Endocrinology. 1998; 139:2423–2431. [PubMed: 9564854]

Jesenberger V, Jentsch S. Deadly encounter: Ubiquitin meets apoptosis. Nat Rev Mol Cell Biol. 2002; 3:112–121. [PubMed: 11836513]

Katychev A, Wang X, Duffy A, Dore-Duffy P. Glucocorticoid-induced apoptosis in CNS microvascular pericytes. Dev Neurosci. 2003; 25:436–446. [PubMed: 14966384]

Kim H, Rafiuddin-Shah M, Tu HC, Jeffers JR, Zambetti GP, Hsieh JJ, Cheng EH. Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. Nat Cell Biol. 2006; 8:1348–1358. [PubMed: 17115033]

Kogianni G, Mann V, Ebetino F, Nuttall M, Nijweide P, Simpson H, Noble B. Fas/CD95 is associated with Glucocortioid-induced osteocyte apoptosis. Life Sci. 2004; 75:2879–2895. [PubMed: 15454340]

Ley R, Balmanno K, Hadfield K, Weston C, Cook SJ. Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein, Bim. J Biol Chem. 2003; 278:18811–18816. [PubMed: 12646560]

Li B, Dou QP. Bax degradation by the ubiquitin/proteasome-dependent pathway: Involvement in tumor survival and progression. Proc Natl Acad Sci USA. 2000; 97:3850–3855. [PubMed: 10725400]

Liang M, Espina B, Russell G, Hulley P. Bim is transcriptionally regulated during induction of apoptosis by growth factor withdrawal in osteoblasts. Calcif Tissue Int. 2007; 80:s81.

Luciano F, Jacquel A, Colosetti P, Herrant M, Cagnol S, Pages G, Auberger P. Phosphorylation of Bim-EL by Erk1/2 on serine 69 promotes its degradation via the proteasome pathway and regulates its proapoptotic function. Oncogene. 2003; 22:6785–6793. [PubMed: 14555991]

Marani M, Hancock D, Lopes R, Tenev T, Downward J, Lemoine NR. Role of Bim in the survival pathway induced by Raf in epithelial cells. Oncogene. 2004; 23:2431–2441. [PubMed: 14676826]

Marchetti MC, Di Marco B, Cifone G, Migliorati G, Riccardi C. Dexamethasone-induced apoptosis of thymocytes: Role of glucocorticoid receptor-associated Src kinase and caspase-8 activation. Blood. 2003; 101:585–593. [PubMed: 12393559]

Memon SA, Moreno MB, Petrak D, Zacharchuk CM. Bcl-2 blocks glucocorticoid-but not Fas- or activation-induced apoptosis in a T cell hybridoma. J Immunol. 1995; 155:4644–4652. [PubMed: 7594463]

O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S, Huang DC. Bim: A novel member of the Bcl-2 family that promotes apoptosis. EMBO J. 1998; 17:384–395. [PubMed: 9430630]

Planey SL, Derfoul A, Steplewski A, Robertson NM, Litwack G. Inhibition of glucocorticoid-induced apoptosis in 697 pre-B lymphocytes by the mineralocorticoid receptor N-terminal domain. J Biol Chem. 2002; 277:42188–42196. [PubMed: 12194973]

Plotkin LI, Weinstein RS, Parfitt AM, Roberson PK, Manolagas SC, Bellido T. Prevention of osteocyte and osteoblast apoptosis by bisphosphonates and calcitonin. J Clin Invest. 1999; 104:1363–1374. [PubMed: 10562298]

Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Mol Cell. 1999; 3:287–296. [PubMed: 10198631]

Reginato MJ, Mills KR, Paulus JK, Lynch DK, Sgroi DC, Debnath J, Muthuswamy SK, Brugge JS. Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. Nat Cell Biol. 2003; 5:733–740. [PubMed: 12844146]

Schacke H, Docke WD, Asadullah K. Mechanisms involved in the side effects of glucocorticoids. Pharmacol Ther. 2002; 96:23–43. [PubMed: 12441176]

Strasser A, Puthalakath H, Bouillet P, Huang DC, O'Connor L, O'Reilly LA, Cullen L, Cory S, Adams JM. The role of bim, a proapoptotic BH3-only member of the Bcl-2 family in cell-death control. Ann N Y Acad Sci. 2000; 917:541–548. [PubMed: 11268382]

Suzuki Y, Nakabayashi Y, Takahashi R. Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death. Proc Natl Acad Sci USA. 2001; 98:8662–8667. [PubMed: 11447297]

Wang Z, Malone MH, He H, McColl KS, Distelhorst CW. Microarray analysis uncovers the induction of the proapoptotic BH3-only protein Bim in multiple models of glucocorticoid-induced apoptosis. J Biol Chem. 2003; 278:23861–23867. [PubMed: 12676946]

Warton K, Foster NC, Gold WA, Stanley KK. A novel gene family induced by acute inflammation in endothelial cells. Gene. 2004; 342:85–95. [PubMed: 15527968]

Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC. Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. J Clin Invest. 1998; 102:274–282. [PubMed: 9664068]

Yamamoto T, Nishiguchi M, Inoue N, Goto HG, Kudawara I, Ueda T, Yoshikawa H, Tanigaki Y, Nishizawa Y. Inhibition of murine osteosarcoma cell proliferation by glucocorticoid. Anticancer Res. 2002; 22:4151–4156. [PubMed: 12553047]

Zalavras C, Shah S, Birnbaum M, Frenkel B. Role of apoptosis in glucocorticoid-induced osteoporosis and osteonecrosis. Crit Rev Eukaryot Gene Expr. 2003; 13:221–235. [PubMed: 14696969]

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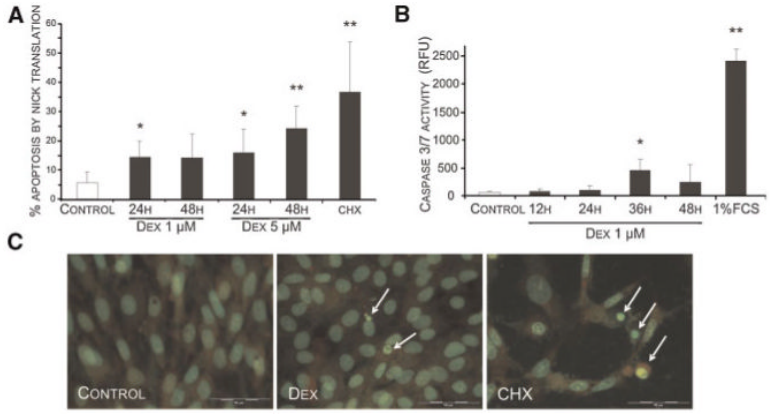

Fig. 1.

Dex induces apoptosis in the mouse osteoblastic cell line MBA 15.4. A: Cells cultured in normal medium and 10% FCS were treated for 24–48 h with Dex (1 and 5 μM). Cycloheximide (CHX) was used as positive control with a dose of 40 μg/ml for 24 h. Apoptosis was measured using Nick Translation assay by flow cytometry. Bars represent mean ± SD of triplicate independent experimental repeats. B: MBA 15.4 cells were treated with Dex 1 μM for different times. Caspase 3/7 activity was measured by release of fluorescence by the cleavage of a profluorescent substrate. Cells maintained in low serum (1%FCS) were used as a positive control. Bars represent mean ± SD of two independent experimental repeats. C: Osteoblastic cells were cultured on coverslips and then treated with vehicle alone or Dex1 μM for 48 h. CHX was used as positive control with a dose of 40 μg/ml for 24h. Cells were fixedin Walpole's buffer followed by staining with acridine orange and apoptotic nuclear morphology was evaluated. RFU, relative fluorescence units. $*P < 0.05$, $**P < 0.005$ versus solvent control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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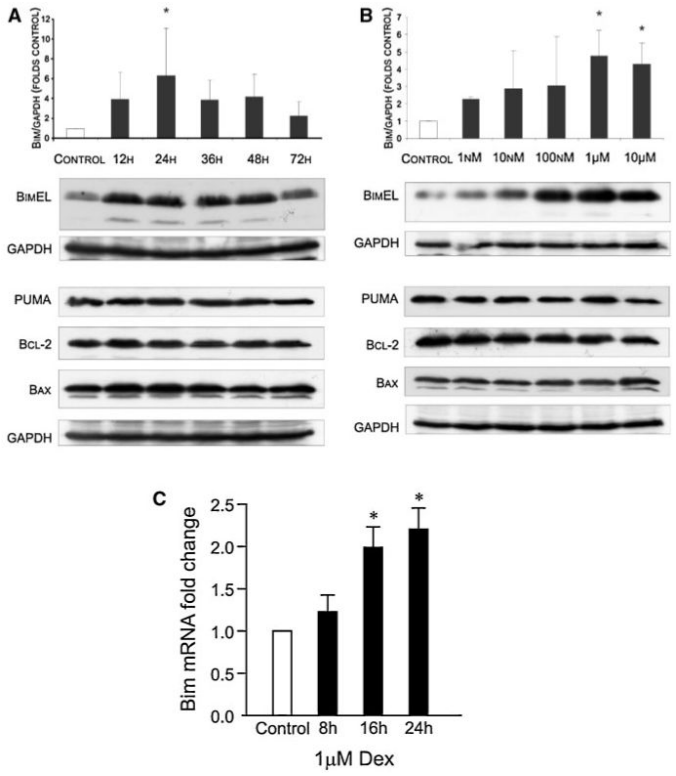

Fig. 2.

Bim induction in MBA 15.4 mouse osteoblastic cells following treatment with dexamethasone. MBA 15.4 cells were treated with Dex 1 μM for the indicated times (A,C) or for 48 h with the indicated range of doses. B: Expression levels of BimEL, PUMA, Bax and Bcl-2 were determined by Western blot analysis (A,B). The protein GAPDH was used as a loading control. The average Bim/GAPDH ratio of triplicate experiments and the fold change are shown. Total RNA was isolated and the expression of Bim RNA was analysed by real-time PCR and normalized to the level of GAPDH (C). The data shown represents mean ± SD of at least three independent experiments. $*P < 0.05$ versus solvent control.

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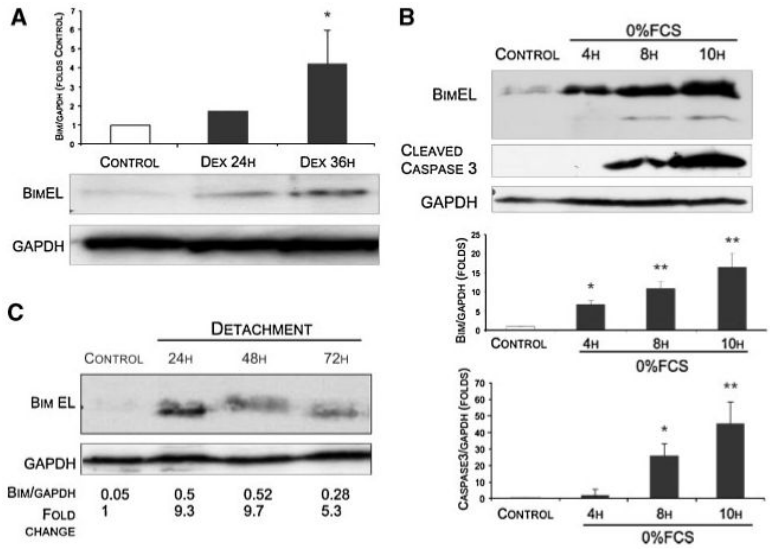

Fig. 3.

A: Human bone marrow stromal cells were treated with Dex 1 μM for the indicated times. Bim was detected by Western blot analysis using GAPDH as the loading control. Bars represent Bim/GAPDH and fold change to the control ±SD from triplicate experiments (Dex 24 h sample was only assayed once). Cells were obtained from a single bone marrow sample. B: MBA 15.4 cells were cultured in 0% FCS for the indicated times. Bim and cleaved Caspase 3 were detected by Western blot using GAPDH as a loading control. Bars represent Bim/GAPDH and Caspase 3/GAPDH fold change to the control ±SD of triplicate experiments. C: MBA 15.4 cells were trypsinised and transferred to agarose coated 10 cm plates for the indicated time periods. Bim levels were quantified by Western blot using GAPDH as a loading control. The average Bim/GAPDH ratio of a representative experiment and the fold changes are shown. $*P < 0.05$, $**P < 0.005$ versus solvent control.

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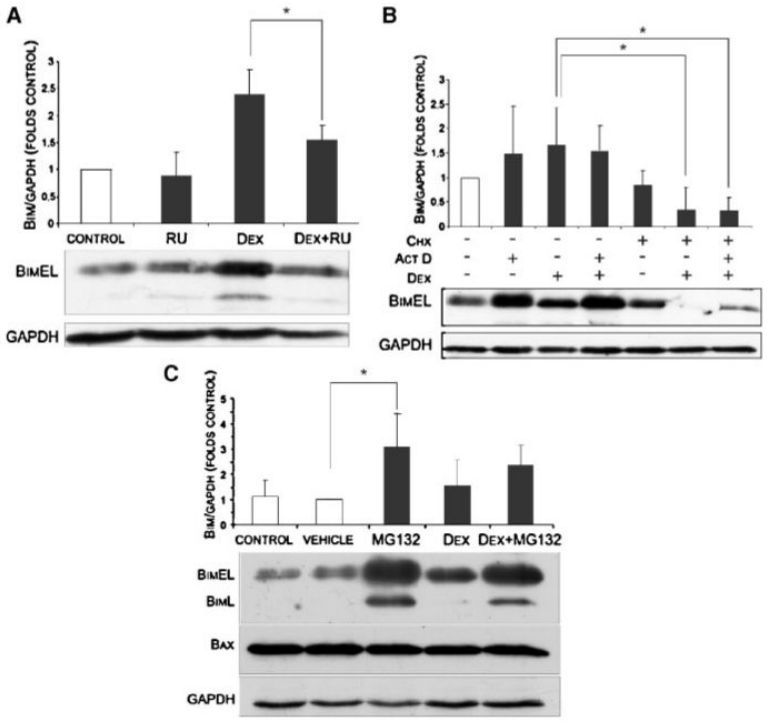

Fig. 4.

A: MBA 15.4 cells were treated with Dex (100 mM) and/or RU486 (1 μM) for 24 h. Bim levels were quantified by Western blot using GAPDH as a loading control. Bars represent Bim/GAPDH fold change to the control ±SD of triplicate experiments. **P* < 0.05 versus dexamethasone alone sample. B: MBA 15.4 cells were treated with Dex (1 μM) and/or CHX (0.5 μg/ml) and/or actinomycin D (10 nM) for 24 h. Bim was detected by Western blot using GAPDH as a loading control. Bars represent Bim/GAPDH fold change to the control ±SD of triplicate experiments. **P* < 0.05 versus dexamethasone alone sample. C: MBA 15.4 cells were treated with the 26S proteasome inhibitor MG132 (1 μM) and/or Dex 1 μM for 24 h. Bim and Bax were detected by Western blot using GAPDH as a loading control. Bars represent Bim/GAPDH fold change to the control ±SD of triplicate experiments. **P* < 0.05 versus solvent control.

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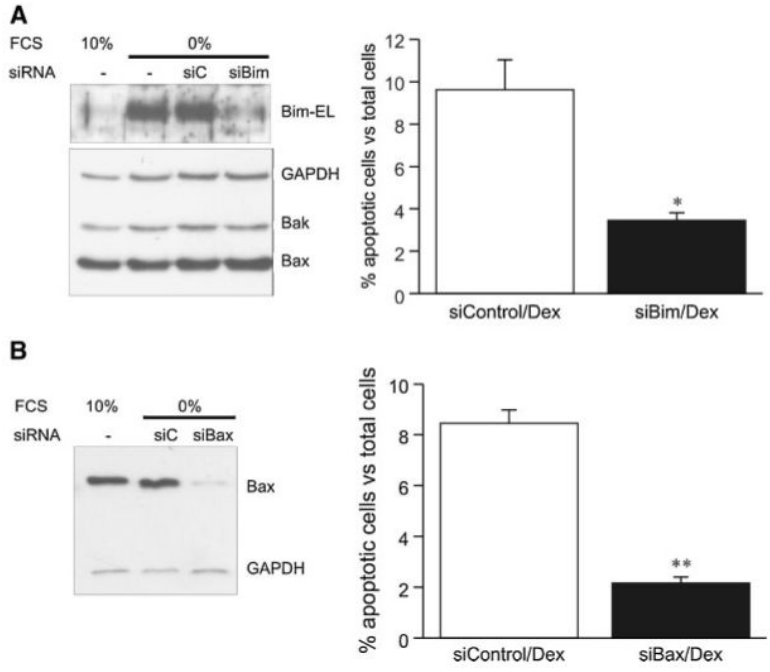

Fig. 5.

The effect of Bim- and Bax-specific siRNA on the induction of apoptosis by dexamethasone treatment of mouse osteoblastic cells. A: left part, optimization of knock-down of Bim protein by RNA interference in MBA 15.4 mouse osteoblastic cells. Cells were transfected with 30 nM siBim or 30 nM siControl and then cultured in 10% FCS until 24 h post-transfection or serum deprived for the last 18 h. Bim EL, Bax and Bak were detected by Western blot using GAPDH as a loading control. A: right part, transfected cells (siControl and siBim) were treated with Dex 1 μM for 24 h. Cells were then fixed and DNA fragmentation investigated by TUNEL in situ assay followed by DAPI staining for total cell counts. TUNEL-positive and normal nuclei were counted. Bars represent mean ± SD of four experiments. B: left part, optimization of knock-down of Bax protein by RNA interference in MBA 15.4 mouse osteoblastic cells, as described above for siBim, except that knock-down of Bax took 48 h. B: right part, transfected cells (siControl and siBax) were treated with Dex 1 μM for 24 h. Apoptotic and normal cells were detected as described above. Bars represent mean ± SD of four experiments. $*P < 0.05$, $**P < 0.005$ versus siControl.

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