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1,25 Dihydroxyvitamin D3 and Dexamethasone Induce the Cyclooxygenase 1 Gene in Osteoclast-Supporting Stromal Cells

Amy E. Adams,[1,2,3]* Yousef Abu-Amer,[4] Jean Chappel,[4] Sharon Stueckle,[1] F. Patrick Ross,[4] Steven L. Teitelbaum,[4] and Larry J. Suva[1]

[1]Division of Bone and Mineral Metabolism, Beth Israel-Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215
[2]Harvard-M.I.T. Division of Health Sciences and Technology, Harvard Medical School, Boston, Massachusetts 02215
[3]Department of Biological Chemistry and Molecular Pharmacology, Biological and Biomedical Sciences Program, Harvard Graduate School of Arts and Sciences, Boston, Massachusetts 02215
[4]Department of Pathology, Washington University School of Medicine, St. Louis, Missouri 63110

Abstract Commitment of members of the monocyte/macrophage family to the bone resorptive phenotype, in vitro, requires contact, of these osteoclast precursors, with osteoblasts or related stromal cells. The osteoclast-inductive properties of these stromal cells are typically expressed, however, only in the presence of steroid hormones such as 1,25 dihydroxyvitamin D (1,25D3) and dexamethasone (DEX). To gain insight into the means by which steroid treated accessory cells induce osteoclast differentiation we asked, using differential RNA display (DRD), if gene expression by this stromal cell population differs from that of their untreated, non-osteoclastogenic counterpart. We identified four known genes specifically expressed by 1,25D3/DEX-treated ST2 stromal cells: 1) a family of rat organic anion transporters, 2) Na/K ATPase ß-subunit, 3) tazarotene-induced gene 2 (TIG2), and 4) prostaglandin G/H synthase I, or cyclooxygenase 1 (Cox-1). The regulation of these genes in 1,25D3/DEX-treated ST2 cells was demonstrated by Northern blot analysis of treated (osteoclast-supporting) and untreated (non-osteoclast-supporting) ST2 cells; the genes have a limited and specific tissue mRNA expression pattern. Northern blot analysis of treated and untreated ST2 cell total RNA using either a DRD-derived Cox-1 cDNA or a Cox-1 specific oligonucleotide confirmed the steroid regulation of Cox-1 mRNA. Surprisingly, there is no detectable expression by untreated or steroid exposed ST2 cells, of Cox-2, the classical regulated cyclooxygenase isoform. In contrast to 1,25D3/DEX, serum treatment rapidly induces Cox-2 mRNA, substantiating the capacity of ST2 cells to express the gene. These data establish that steroid induction of the osteoclastogenic properties of stromal cells is attended by Cox gene expression, a phenomenon consistent with the capacity of eicosinoids to impact the resorptive process. The response of osteoclast-supporting ST2 cells to 1,25D3/DEX treatment may be one prostaglandin-mediated event which specifically involves Cox-1 regulation. J. Cell. Biochem. 74:587–595, 1999.

Key words: steroid hormones; stromal cells; osteoclastogenesis; cyclooxygenase

Bone is a complex tissue in which resorption and formation occur in a coordinated fashion termed remodeling [Parfitt, 1988]. Bone remodeling is regulated by the action of certain osteotropic hormones, such as 1,25 dihydroxyvitamin D_3 (1,25D3), dexamethasone (DEX), parathyroid hormone (PTH), and calcitonin (CT) which modulate bone resorption via specific effects on osteoclast function [Roodman, 1996; Takahashi et al., 1988].

Osteoclasts are polarized, multinucleated bone resorptive cells derived from hemopoietic precursors, recruited to the skeleton from the marrow and circulation. During the complex process of osteoclast differentiation, mononuclear progenitors proliferate and differentiate into preosteoclasts which eventually fuse to form functional bone-resorbing polykaryons

L.J. Suva's current address is Bone and Cartilage Biology, Smith Kline Beecham Pharmaceuticals, 709 Swedeland Rd., King of Prussia, PA 19406.

*Correspondence to: Amy E. Adams, Beth Israel-Deaconess Medical Center, HIM 944, 330 Brookline Ave., Boston MA 02215. E-mail: aadams@bidmc.harvard.edu

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[Roodman, 1996]. Although this cascade is morphologically well-defined, the specific molecular events regulating osteoclastogenesis remain incompletely understood.

On the other hand, the development of systems whereby bone fide osteoclasts may be generated, in vitro, has added substantially to delineating the nature of osteoclast precursors and the means by which they differentiate into mature resorptive cells. For example, it is now known that commitment to the osteoclast phenotype, by mammalian mononuclear precursors, requires their contact with mesenchymal cells, either osteoblasts or related marrow-derived stromal cells [Jimi et al., 1996].

Steroid hormones play a pivotal role in osteoclast differentiation, as optimal generation of resorptive polykaryons requires 1,25D3 and, often, Dex [Kurihara et al., 1989]. While these steroids may impact osteoclast precursors, they target the supportive stromal cell population [Udagawa et al., 1989; Suda et al., 1992; Matsumoto et al., 1995]. The precise steroid-activated stromal cell genes which promote osteoclastogenesis are, however, unknown.

Using a differential mRNA display (DRD) approach [Liang and Pardee, 1992] comparing mRNAs expressed by ST2 stromal cells induced to be osteoclastogenic by 1,25D3 and DEX, to mRNAs of their non-steroid treated, and thus non-osteoclastogenic, counterparts we find that Cox-1, but not Cox-2, is included among those genes specifically expressed by steroid-treated ST2 cells. While these data are reflective of the potent osteoclastogenic properties of Cox products, induction by 1,25/DEX treatment of only Cox-1, the isoform believed to be constitutive, provides a potentially novel means of regulating osteoclastogenesis.

MATERIALS AND METHODS

Materials

1,25 dihydroxyvitamin D_3 (1,25D3) was a gift of Dr. Milan Uskokovic (Hoffmann La-Roche, Nutley, NJ), and dexamethasone (DEX) was purchased from Sigma (St. Louis, MO). All cell culture media, serum and plasticware were purchased from Gibco-BRL (Gaithersburg, MD).

Cell Culture and Treatments

ST2 cells were plated with α-MEM supplemented with 10% heat-inactivated fetal bovine serum at cell density of 10^4 (10,000) cells/cm^2/ml and were maintained at 37°C in 5% CO2. At ~70% confluence in α-MEM supplemented with 10% FBS and then the media was changed to α-MEM supplemented with 10% charcoal-stripped FBS with or without the addition of 10^{-8} M 1,25D3 and 10^{-7} M DEX [Abu-Amer et al., 1997]. For serum stimulation, cells grown in 10 cm^2 tissue culture dishes were rinsed twice with serum-free α-MEM and grown 16 h in α-MEM supplemented with 1% FBS. Cells were then treated with α-MEM supplemented with 10% FBS. Cells were harvested by trypsin treatment 1 h and 4 h post serum-stimulation, and control cells prior to serum stimulation.

RNA Preparation and Northern Analysis

Total cellular ST2 RNA was isolated by guanidinium isothiocyanate and phenol extraction as previously described [Adams et al., 1995]. Total RNA (20 μg/well) was separated by electrophoresis through a 1.5% agarose gel containing 0.66 M formaldehyde and transferred to nylon (Hybond N, Amersham, Arlington Heights, IL). Filters containing ST2 total RNA with and without 1,25D3/DEX treatment, and filters containing ST2 cell total RNA isolated from cells with and without serum starvation were prehybridized in ExpressHyb hybridization solution (Clontech, Palo Alto, CA) at 68°C and then hybridized in the same buffer containing the ^{32}P-labeled DRD-derived cDNA fragment as a probe (~10^6 cpm/ml) for 1 h. Filters were washed three times at room temperature in 2× SSC/0.1% SDS and two times at 50°C in 0.1× SSC/0.1% SDS. Murine multiple tissue Northern blots (Clontech) were also screened as described above. cDNA fragments for use as probes were generated by EcoRI restriction enzyme digestion, and subsequent extraction from agarose gels using Geneclean (Bio 101 Inc., Vista, CA). Specific oligonucleotides to Cox-1 and Cox-2 mRNA (purchased from Oxford Biomedical, Oxford, MI) were radiolabeled using T4 polynucleotide kinase (Boehringer Mannheim, Germany) and α-^{32}P ATP as described [Suva et al., 1994]. Fold increases in mRNA expression were estimated using densitometry of autoradiographs.

RNA Differential Display

Fifty microgram samples of ST2 cell total RNA (48 h 1,25D3/DEX treatment and untreated control) were DNAse treated using Mes-

sageclean (GeneHunter, Nashville, TN) and DRD performed as described [Liang and Pardee, 1992]. Briefly, samples were digested for 30 min with DNAse I at 37°C, phenol/chloroform extracted and precipitated with ethanol and 0.3 M NaOAc, and resuspended in DEPC-treated water. Using RNAmap (GeneHunter), samples were reverse transcribed with minor modifications to the manufacturers protocol as follows: RNA samples were freshly diluted to 0.1 µg/µl. Reverse transcription reactions were set up for each RNA sample in four sterile RNA tubes. The following core mixes for each of the T12 N oligonucleotide primers were prepared for a 20 µl final volume (9.4 µl dH2O, 4 µl 5× RT buffer, 1.6 µl dNTP (250 µM stock), 2 µl T12 N [N= A, G or C] (10 µM stock). Samples were incubated in a Perkin-Elmer 9600 thermocycler (Perkin Elmer, CA) as follows: 65°C, 5 min; 37°C, 10 min; pause 10 min and add 1 µl MMLV reverse transcriptase to each tube; 37°C, 50 min; 95°C, 5 min; hold at 4°C. Tubes were then microcentrifuged and stored at -20°C for later use. For polymerase chain reaction (PCR), the following core mixes were prepared for a 20 µl final volume (9.2 µl dH2O, 2.0 µl 10× PCR buffer, 1.6 µl dNTPs (25 µM stock) 1 µl α^{35}S-dATP (1,200 Ci/mmole), 0.2 µl AmpliTaq (Perkin-Elmer). Fourteen µl of the core mix was aliquoted into thin-walled PCR tubes and 2 µl of each T12 N oligonucleotide primer added to three tubes/RNA sample. Two µl of reverse transcription reaction was added to each tube (1 h treated or control RNA). To one of each reaction tube, 2 µl of arbitrary primer (AP; 2 µM stock) was added. A total of 80 arbitrary primers were used. Samples were mixed, and incubated in a Perkin-Elmer 9600 thermocycler as follows: 94°C, 30 sec; 40°C, 2 min; 72°C, 30 sec for 40 cycles; 72°C, 5 min; hold at 4°C. 3.5 µl of each sample was mixed with 2 µl loading dye, incubated for 2 min at 80°C, and resolved in parallel lanes on a 6% non-denaturing (native) polyacrylamide gel in 1× TBE. Gels were run for 3.5 h in 1× TBE running buffer at 60W constant power. Gels were blotted to 3M paper, dried, and exposed at room temperature to Kodak Biomax film (Rochester, NY) with two intensifying screens for 24 h and 72 h. Bands were identified as putatively regulated in treated vs. control lanes and the DRD repeated with the same AP and T12N primers, using new reverse transcription reactions. Those bands which were found to be consistently upregulated in three DRD protocols were excised from the dried gel. The DNA was eluted in 100 µl dH2O, decanted from the gel/paper debris, and precipitated with ethanol and 0.3M sodium acetate in the presence of glycogen. The cDNA pellets were washed with 85% EtOH and resuspended in 10 µl dH2O. Four µl of each cDNA was used for reamplification. Forty µl reamplification reactions were performed using the same primer set and PCR conditions, except that the dNTP stock was 250 µM (for a 20 µM final concentration), and no radioisotope was used. Re-amplified samples were run on 1.5% agarose gels, and bands excised and extracted using Geneclean. Samples were cloned into the pCRII T/A cloning vector (Invitrogen, San Diego, CA) and transformed into E. coli DH5α (BRL). Plasmid DNA was isolated and purified (Qiagen) and completely sequenced using Sequenase (USB, Cleveland, OH).

Sequence Analysis

Sequences were compared to Genebank, using Blast [Altschul et al., 1990].

RESULTS

To compare mRNAs induced by osteoclast supporting and non-supporting accessory cells, total RNA was isolated from ST2 cells exposed to 1,25D3/DEX or vehicle, respectively, for 48 h at which time specific markers of generated osteoclasts appear [Suda et al., 1992]. Various combinations of cDNAs were compared by DRD, using 240 primer pairs. We thus identified five genes in steroid treated cells, specific upregulation of which was confirmed by cDNA reamplification and Northern analysis of ST2 cell RNA (Fig. 1). The complete nucleotide sequences of the identified DRD-derived, steroid-induced cDNA fragments were compared to the Genbank database [Altschul et al., 1990] and one novel and four characterized potential genes were discovered. The identified cDNAs were then used to probe murine tissue Northern blots (Clontech) (Fig. 2). The mRNA recognized by Clone G28 cDNA, homologous to a rat organic anion exchanger (OAT-K1) [Saito et al., 1996], exhibits restricted distribution, as the major ~4.0 kb transcript is detectable predomainantly in brain (Fig 2B). Three of the remaining mRNAs are more ubiquitous, although none is present in all tissues examined.

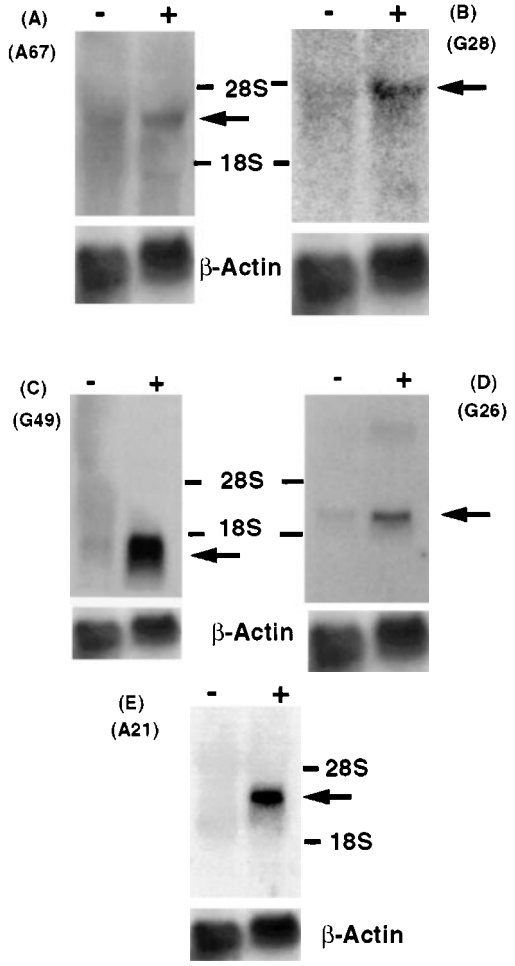

Fig. 1. Northern blot of ST2 mRNA with DRD-derived clones. **A**: DRD-derived clone A67; **B**: DRD-derived clone G28; **C**: DRD-derived clone G49; **D**: DRD-derived clone G26; **E**: DRD-derived clone A21. Each clone was hybridized to ST2 RNA from cells treated without (-) or with (+) 1,25D3/DEX treatment for 2 days. 28S and 18S RNA are shown as size markers. Arrows indicate the size of the individual mRNA transcripts. β-actin hybridization of the blots are shown below as a loading control.

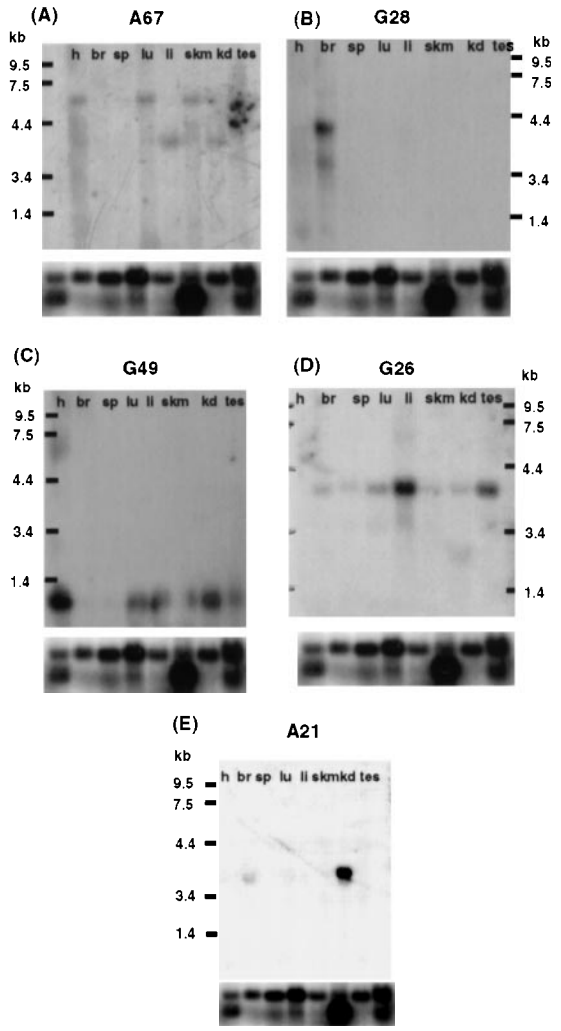

Fig. 2. Tissue distribution of DRD-derived clones. **A**: DRD-derived clone A67; **B**: DRD-derived clone G28; **C**: DRD-derived clone G49; **D**: DRD-derived clone G26; **E**: DRD-derived clone A21. Each clone was hybridized with mRNA from a variety of murine tissues. h, heart; br, brain; sp, spleen; lu, lung; li, liver; skm, skeletal muscle; kd, kidney; tes, testis. Size markers are shown in kb. β-actin hybridization of the blots are shown below as a loading control.

We also established that the Na/K ATPase ß-subunit (accession X61433), clone A21 (Fig 1E), as well as a novel gene (clone A67; Fig 1A) are regulated by 1,25/DEX. Clone A67 mRNA is widely distributed, with the ~6 kb transcript most abundant in heart and lung and a ~4 kb transcript in liver and kidney (Fig. 2A). Clone G49, identified as tazarotene-induced gene 2 (TIG2) [Nagpal et al., 1997] and G26, identical to the cyclooxygenase-1 gene (Cox-1) [DeWitt and Smith, 1988], were selected for further analysis.

TIG2 is a recently identified retinoic acid (RA)-regulated human gene whose skin expression is not known to be impacted by other steroids [Nagpal et al., 1997]. As shown in Figure 1C, murine TIG2 mRNA (~0.8 kb) is dramatically induced in ST2 cells by 1,25D3/DEX. With two days exposure to 1,25D3/DEX TIG 2 is induced ~20-fold, returning to basal levels by day 5 (Fig. 3). Similarly, the 2.1 kb G26 (Cox-1) transcript is upregulated ~10-fold in 1,25D3/DEX-treated ST2 cells and virtually unaltered in control cells. Confirming this observation, Northern analysis using a Cox-1 specific oligonucleotide reveals induced expression of a 2.1 kb mRNA transcript, identical in size to that observed with clone G26 cDNA (Fig. 4). Interest-

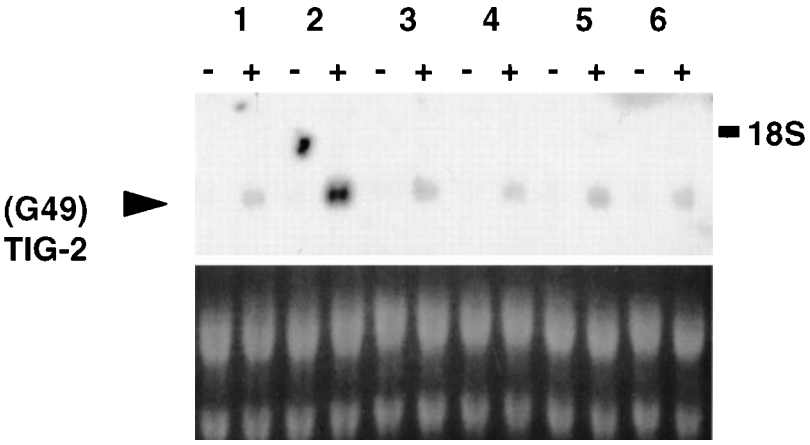

Fig. 3. Time-course of G49 (TIG-2) mRNA expression in 1,25D3/DEX-treated ST2 cells. Total RNA from ST2 cells untreated (-) or treated (+) with 1,25D3/DEX for days 1–6 of culture were screened with DRD-derived G49 cDNA. The position of the 0.8 kb mRNA transcript is shown by the arrow. The position of 18S rRNA is shown as a size marker. Ethidium bromide staining of the original gel is shown as a loading control.

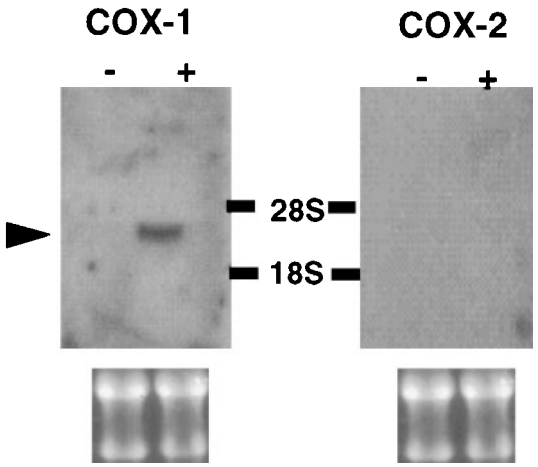

Fig. 4. Identification of regulated COX-1 expression in ST2 cells. Total RNA from ST2 cells untreated (-) or treated (+)with 1,25D3/DEX for 2 days was screened with a Cox-1-specific oligonucleotide (COX-1) or Cox-2-specific oligonucleotide (COX-2). The arrow shows the position of the ~2.1 kb Cox-1 mRNA, identical to that seen with DRD-derived clone G26 (Fig. 1D). No expression or regulation of Cox-2 was observed. The position of 28S and 18S rRNA size markers are shown. Ethidium bromide staining of the original gel is shown as a loading control.

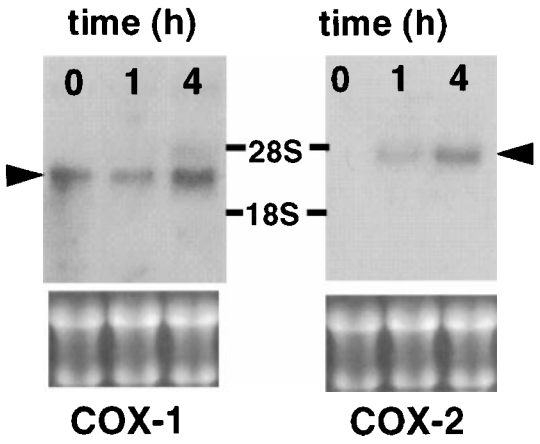

Fig. 5. Serum-induction of Cox-1 and Cox-2 mRNA in ST2 cells. Total RNA from serum-deprived ST2 cells stimulated with serum for 1 and 4 hours and probed with Cox-1 (COX-1) or Cox-2 (COX-2) specific oligonucleotides. Arrows show the position of the Cox-1 and Cox-2 mRNA transcripts. The position of 28S and 18S rRNA size markers are shown. Ethidium bromide staining of the original gel is shown as a loading control.

ingly, no Cox-2 mRNA transcript is detected in either treated or control ST2 cell RNA (Fig. 4).

In order to determine if ST2 cells are capable of expressing Cox-2 mRNA, we asked if Cox-2 gene expression is stimulated by serum (Fletcher et al., 1992). As seen in Figure 5, exposure of serum-starved (1% FBS for 24 h) ST2 cells to serum (10% FBS) induces both Cox isoforms but at distinctly different rates. Whereas Cox-1 mRNA is moderately induced after 4 h of serum stimulation (Fig. 5), only Cox-2 message appears within 1 h (Fig. 5). Thus, while ST2 cells have the capacity to regulate both isoforms, 1,25D3/DEX treatment specifically enhances Cox-1 mRNA expression.

1,25D3/DEX treatment of ST2 cells generally prompts appearance of characteristic osteoclasts by day 6 [Suda et al., 1992]. In keeping with a potential and specific role of COX 1 in the osteoclastogenic process, the isoform is upregulated in ST2 cells by day 2 of steroid exposure, returning to basal levels by day 4 (Fig. 6), consistent with the time course of osteoclastogenesis. Cox-2 mRNA, in contrast, fails to appear within this time, in treated or untreated cells (data not shown). Thus, 1,25D3/DEX specifically induces the expression, by ST2 cells, of the Cox-1, but not Cox-2 gene. Additionally, Cox-1 mRNA levels in ST2 cells exposed to either steroid for 48 h are relatively modest as

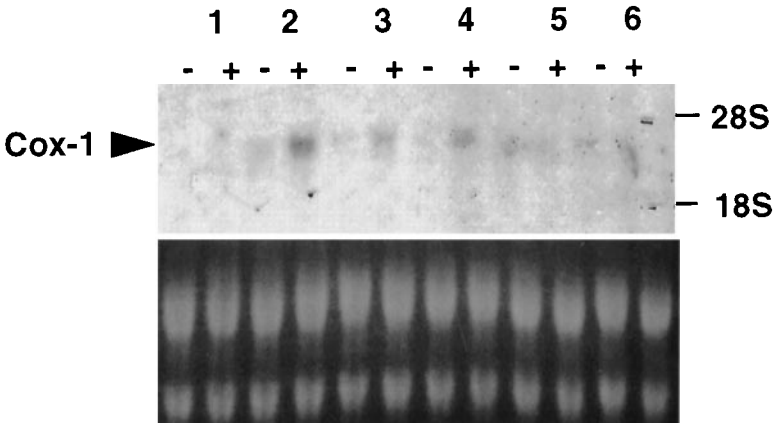

Fig. 6. Time-course of Cox-1 mRNA expression in 1,25D3/DEX-treated ST2 cells. Total RNA from ST2 cells untreated (-) or treated (+) with 1,25D3/DEX for days 1–6 of culture were screened with a Cox-1-specific oligonucleotide. The position of the ~2.1 kb Cox-1 mRNA transcript is shown by the arrow. The position of 28S and 18S rRNA are shown as size markers. Ethidium bromide staining of the original gel is shown as a loading control.

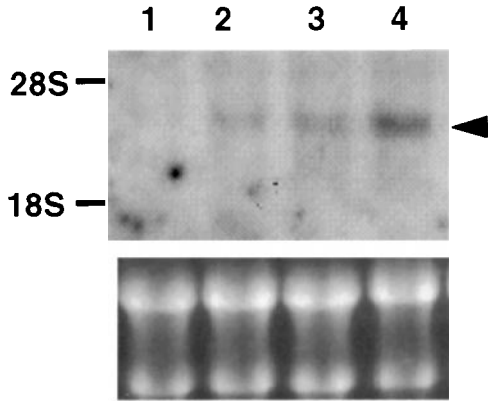

Fig. 7. Northern blot of ST2 cell total mRNA screened with a Cox-1-specific oligonucleotide. **Lane 1**: Control ST2 RNA. **Lanes 2–4**: RNA from ST2 cells treated for 2 days with (2) 1,25D3, (3) DEX, (4) 1,25D3/DEX. The position of the ~2.1 kb Cox-1 mRNA transcript is shown by the arrow. The position of 28S and 18S rRNA are shown as size markers. Ethidium bromide staining of the gel is shown as a loading control.

compared to those in cells maintained with both 1,25D3 and DEX (Fig. 7).

DISCUSSION

Various mesenchymal cells, including those derived from marrow stroma [Hattersley et al., 1991; Udagawa et al., 1989; Yamashiya et al., 1990] primary osteoblasts, osteoblast-like cells [Matsumoto et al., 1995; Yamashiya et al., 1990] as well as the pluripotent embryonic cell line C3H10T1/2, support osteoclast formation, in vitro. With the recent discovery of osteoprotogerin ligand (RANKL) as a ST2 cell residing, osteoclast inductive protein [Lacey et al., 1998; Yasuda et al., 1998], the phenotype of osteoclastogenic supportive cells is beginning to emerge.

In the present study we identified five genes specifically induced, in ST2 cells, by the osteoclastogenic steroids, 1,25 D3 and DEX. Of the five, two code for ion regulators [Jacquemin et al., 1994; Saito et al., 1996] whose function in osteoclast recruitment, given present information, is arcane. It is of interest, however, that oxygen free radicals stimulate osteoclast formation [Garrett et al., 1990], raising the possibility that microenvironment ion concentrations may modulate osteoclastogenesis.

Murine homologs of the previously identified TIG2, and Cox-1 genes [Dewitt and Smith, 1988; Nagpal et al., 1997] represent the two remaining ST2 genes induced by 1,25D3/DEX treatment. TIG2 encodes a putative 164 amino acid protein [Nagpal et al., 1997] of unknown function. The 0.8 kb TIG2 mRNA transcript was initially identified by subtraction hybridization screening of retinoid-treated human skin graft cultures, devlopment of which requires contact between keratinocytes and fibroblasts [Nagpal et al., 1997]. TIG2 mRNA is neither expressed nor retinoic acid-regulated in cultures containing only primary keratinocytes or fibroblasts, suggesting modulation of the gene requires a tissue-like, three-dimensional environment [Nagpal et al., 1997]. Because TIG2 regulation in skin culture is retinoid limited, our observations with ST2 cells suggests specific steroids modulate the gene on a cell selective basis.

Cox's are enzymes central to conversion of membrane-bound arachidonic acid to prostaglandins [Kawaguchi et al., 1995]. The Cox-1 and 2 isoforms are encoded by distinct genes located on different chromosomes [Wen et al., 1993]. Not surprisingly, therefore, transcriptional regulation of Cox-1 and Cox-2 differs and the two isoforms appear to enjoy distinct properties [Langenbach et al., 1995; Morita et al., 1995; Murakami et al., 1994]. As it is expressed constitutively in many tissues [Langenbach et

al., 1995; Simmons et al., 1989], and traditionally not believed to mediate stimulated biological events, Cox-1 has been considered a "housekeeping" gene [De Witt and Smith, 1988; Langenbach et al., 1995; Simmons et al., 1989]. Recently, however, the isoform has proven, in some circumstances, to be regulated [Samet et al., 1995; Smith et al., 1993, 1996; Toth et al., 1996]. In endothelial cells, for example, cox-1 levels mirror the state of differentiation [Samet et al., 1995]. Cox-1 gene expression is responsive to changes in blood flow, and to cytokine or tumor promoter stimulation [Samet et al., 1995; Smith et al., 1993, 1996]. Cox-1 mRNA is also regulated by human chorionic gonadotropin in human fetal membranes [Toth et al., 1996] and, as established in this study, in marrow stromal cells by 1,25D3/DEX treatment. Cox-2 mRNA and protein are, in contrast, virtually undetectable in most tissues, yet induced by many agents such as tumor promoters, cytokines, and mitogens [DuBois et al., 1994; Fletcher et al., 1992]. By virtue of its rapid induction, Cox-2 is considered an immediate early response gene [Fletcher et al., 1992; Langenbach et al., 1995; Simmons et al., 1989].

While prostaglandins of the E series promote osteoclastogenesis [Akatsu et al., 1989; Kawaguchi et al., 1995; Suda et al., 1992], the Cox gene responsible for prostaglandin expression during osteoclast development is unknown. Cox-2 is regulated, in bone marrow macrophages and primary osteoblasts, whether cultured alone [Chen et al., 1997; Sato et al., 1996] or in combination [Tai et al., 1997], by osteoclastogenic cytokines such as interleukins. Despite these observations, and the broad functional categorization of Cox-1 ("constitutive") and Cox-2 ("inducible"), our DRD and Northern blot analysis clearly document that only the former is induced in ST2 cells by 1,25D3/DEX. These data raise the possibility that the capacity of these steroids to stimulate prostaglandin expression, by marrow stromal cells [Chen et al., 1997], is mediated by the type 1 isoform.

While 1,25D3 and DEX each independently induce Cox-1 mRNA, their activities are synergistic. This observation is coordinate with the limited capacity of 1,25D3 alone to stimulate osteoclastogenesis in co-culture, a phenomenon greatly enhanced by the presence of DEX [Suda et al., 1992]. Additionally, Cox-1 mRNA expression is stimulated by 1,25D3/DEX in both "young" (passage 13) and "old" (passage 55) ST2 cultures (data not shown), suggesting induction of the isoform does not represent steroid-accelerated cell differentiation.

Prostaglandins of the E series which are synthesized by osteoblasts under the aegis of a variety of growth factors and hormones [Kawaguchi et al., 1995], are complex regulators of bone metabolism, stimulating both resorption [Akatsu et al., 1991; Raisz et al., 1989] and formation [Marks et al., 1989]. Consequently, the presence of multiple pathways prompting Cox gene expression, would not be surprising. This conclusion is in keeping with the regulation of Cox-2 by osteoclastogenic stimuli other than 1,25D3/DEX [Chen et al., 1997; Sato et al., 1996; Tai et al., 1997]. Taken collectively, these data raise the possibility that induction of prostaglandin synthesis by distinct osteoclastogenic stimuli may involve cell and/or tissue-specific regulation of both the Cox-1 and Cox-2 genes.

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