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Inflammatory T Cells Rapidly Induce Differentiation of Human Bone Marrow Stromal Cells Into Mature Osteoblasts

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Abstract Activated T cells secrete multiple osteoclastogenic cytokines which play a major role in the bone destruction associated with rheumatoid arthritis. While the role of T cells in osteoclastogenesis has received much attention recently, the effect of T cells on osteoblast formation and activity is poorly defined. In this study, we investigated the hypothesis that in chronic inflammation activated T cells contribute to enhanced bone turnover by promoting osteoblastic differentiation. We show that T cells produce soluble factors that induce alkaline phosphatase activity in bone marrow stromal cells and elevated expression of mRNA for Runx2 and osteocalcin. This data indicate that T cell derived factors have the capacity to stimulate the differentiation of bone marrow stromal cells into the osteoblast phenotype. RANKL mRNA was undetectable under any conditions in highly purified bone marrow stromal cells. In contrast, RANKL was constitutively expressed in primary osteoblasts and only moderately up-regulated by activated T cell conditioned medium. Interestingly, both bone marrow stromal cells and osteoblasts expressed mRNA for RANK, which was strongly up-regulated in both cell types by activated T cell conditioned medium. Although, mRNA for the RANKL decoy receptor, osteoprotegerin, was also up-regulated by activated T cell conditioned medium, it's inhibitory effects may be mitigated by a simultaneous rise in the osteoprotegerin competitor TNF-related apoptosis-inducing ligand. Based on our data we propose that during chronic inflammation, T cells regulate bone loss by a dual mechanism involving both direct stimulation of osteoclastogenesis, by production of osteoclastogenic cytokines, and indirectly by induction of osteoblast differentiation and up-regulation of bone turnover via coupling. J. Cell. Biochem. 88: 650–659, 2003.
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Key words: osteoblasts; osteoclasts; bone marrow stromal cells; T cells; Runx2

The bone loss, which leads to fracture in inflammatory diseases such as rheumatoid arthritis (RA), is a serious clinical problem affecting 7.5 million people in the US alone. Although significant progress has been made in elucidating the nature of the disease process, the etiology remains elusive. RA is a disease characterized by a chronic proliferative and erosive synovitis that waxes and wanes over the lifetime of the affected individual, with a progressive course. The main characteristics of RA is the dense lymphoid infiltration into the synovial membrane associated with juxta-articular bone loss occurring around inflamed joints and a generalized bone loss with reduced bone mass [reviewed in Gough et al., 1994; Dequeker et al., 1995; Deodhar and Woolf, 1996]. However, RA is also considered a systemic disease which includes serological and hematological signs of inflammation, subcutaneous rheumatoid nodules, high levels of circulating rheumatoid factor, vasculitis, and granulomatous and/or interstitial organ pathologies [Kinne et al., 1997]. Of interest is the fact that 70–80% of RA patients display an association with HLA-DR or HLA-DQ loci of the MHC-II complexes, with predominance of certain alleles that act as independent susceptibility genes. The presence of these MHC-II complexes

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is the most compelling evidence to support the concept that T cell recognition is important at some stage in the pathogenesis of RA, either in shaping the T cell receptor (TCR) or in the presentation of an inducing autoantigenic peptide via an antigen presenting cell [Kinne et al., 1997]. In untreated RA patients, serum markers of bone formation (osteocalcin (OCN) and bone-specific alkaline phosphatase) and urinary markers of bone resorption (procollagen type I C-terminal telopeptide, pyridinoline and deoxypyridinoline, and hydroxyproline excretion) are significantly higher than those of control patients [Gough et al., 1994; Hanna et al., 1997; Suzuki et al., 1998]. These biochemical data are consistent with high bone turnover in RA suggesting that the inflammatory process induces enhanced osteoclastic and osteoblastic activity. The data are also consistent with the notion that osteoclastic activation, rather than suppression of bone formation, is the dominant process leading to bone loss in early RA [Gough et al., 1998]. In this regard, activated T cells have been identified as crucial in the bone loss associated with RA in animal models [Kong et al., 1999]. Although many of these observations stem from the examination of individual specific cytokines secreted from T cells, none examine the effect of the total T cell cyokine repetoire, the conditions that are present under pathological conditions. Therefore, we tested the hypothesis that cytokines secreted by activated T cells regulate the high bone turnover seen in RA through the rapid differentiation of pre-osteoblasts and activation of osteoblasts that inhabit the bone-immune interface in the bone marrow. We now show that during chronic inflammation, activated T cells produce a variety of cytokines which induce human bone marrow stromal cells (BMSC) to differentiate into phenotypic osteoblasts by regulating the expression of Runx2, alkaline phosphatase, and OCN. Thus we propose that T cells regulate bone loss by a dual mechanism involving both direct stimulation of osteoclastogenesis, by the production of osteoclastogenic cytokines, and indirectly by induction of osteoblast differentiation and up-regulation of bone turnover via coupling.

MATERIALS AND METHODS

All reagents were obtained from Sigma Chemical Co (St. Louis, MO) unless otherwise indicated. Use of human tissues and buffy coats was approved by the Human Studies Committee, Washington University Medical Center.

Antibodies and Recombinant Cytokines

Mouse anti-human CD3 monoclonal antibody (clone HIT3a) and mouse anti-human CD28 monoclonal antibody (clone 28.2) were obtained from Pharmingen (San Diego, CA) as sterile, azide free, and low endotoxin preparations.

T Cell Cultures

Peripheral blood mononuclear cells (PBMC) were obtained in the form of buffy coats from the American Red Cross (St. Louis, MO) and further purified by separation on Histopaque (1.077 g/ml) lymphocyte separation medium as previously described [Rifas and Avioli, 1999]. T cells were isolated by positive selection from the PBMC using CD4+ DynaBeads (Dynal Biotech, Lake Success, NY) according to the manufacturer's instructions. This process results in a population of T cells >95% pure. T cells were cultured at 1×10^6 cells/ml in AIM V serum-free medium (Invitrogen, Carlsbad, CA) and activated as previously described [Rifas and Avioli, 1999]. After a 72-h incubation period the activated T cell conditioned media (ACTTCM) were harvested and frozen at -80°C until used in the experimental protocols.

Preparation of Human Osteoblast (hOB) Cultures

Human osteoblast cultures were prepared as previously described [Rifas et al., 1994; Rifas and Avioli, 1999] from rib specimens obtained from the Missouri Transplantation Services (St. Louis, MO) as donor tissue. These cells have the characteristics of osteoblasts as previously reported [Rifas et al., 1989, 1994, 1995].

Bone Marrow Stromal Cells

Bone marrow stromal cells were prepared as previously described [Cheng et al., 1994; Rifas et al., 1995].

Alkaline Phosphatase Assays

Cell layers were washed three times with Tris-buffered saline (TBS), pH 7.4, then incubated in 0.1% SDS for 1 h. The solubilized cell layer was scraped into the SDS and drawn several times though a micropipete tip. Protein was determined using the Bio-Rad DC protein

assay kit (Bio-Rad laboratories, Hercules, CA). Alkaline phosphatase (AP) was measured biochemically using a kit from Sigma. Results were calculated as nmol PNP cleaved/min/mg protein using para-nitro phenol as a standard.

Semi-Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Human osteoblasts were seeded into 100 mm culture dishes (1×10^6 cells/dish) and incubated for 72 h with either medium alone or 25% ACTTCM. Media were aspirated and total RNA extracted using a Qiagen RNeasy mini-kit (Qiagen, Inc., Valencia, CA).

RT-PCR was performed with an RT-PCR ready-to-go kit (Amersham Biosciences). Amplification was performed on 2 μg total RNA per sample using the following primers: Runx2: Forward (5′-TCT TCA CAA ATC CTC CCC-3′), Reverse (5′-TGA TTA AAA GGA CTT GGT G-3′) [Christiansen et al., 2000] hRANK; Forward (5′-GGG AAA GCA CTC ACA GCT AAT TTG-3′), Reverse (5′-GCA CTG GCT TAA ACT GTC ATT CTC C-3′) [Thomas et al., 1999], hRANKL; Forward (5′-TGG ATC ACA GCA CAT CAG AGC AG-3′), Reverse (5′-TGG GGC TCA ATC TAT ATC TCG AAC-3′) [Thomas et al., 1999], hOPG; Forward (5′-GGG GAC CAC AAT GAA CAA GTT G-3′), Reverse (5′-GCT TGC ACC ACT CCA AAT CC-3′) [Thomas et al., 1999], OCN; Forward (5′-CAT GAG AGC CCT CAC A-3′), Reverse (5′-CAG TCC CAC AGC GAG A-3′) [Rickard et al., 1996], hTRAIL; Forward (5′-ATG GGA CTT TGG GGA CAA AGC GTC C-3′), Reverse (5′-CAA GGT GCT GAT GTC AGC GGA GTC A-3′). GAPDH; Forward (5′-GGG CTG CTT TTA ACT CTG GT-3′), Reverse (5′-TGG CAG GTT TTT CTA GAC GG-3′) [Hay et al., 2000] was used as a control to insure that equal amounts of RNA were amplified.

RT was performed at 42°C for 30 min, using $pd(T)_{12-18}$ to generate first strand cDNA, followed by 95°C for 5 min to inactivate the reverse transcriptase. Forward and reverse primers were added and PCR was performed as follows: denaturation 94°C, 1 min; annealing, 60°C, 1 min; elongation, 72°C, 2 min. Negative controls were performed by inactivating the reverse transcriptase by boiling for 5 min prior to RT-PCR to insure that genomic DNA was not being amplified. Runx2, RANK, RANKL, and OCN were amplified for 35 cycles, TRAIL and OPG for 30 cycles, and GAPDH for 20 cycles. All were confirmed to be within the linear range of amplification. PCR products (10 μl) were electrophoresed on 2% agarose E-gels (Invitrogen) containing ethidium bromide, according to the manufacturer's instructions.

Densitometry

E-gel images were scanned using a Hewlett-Packard scanner (Palo Alto, CA) and individual bands quantitated using TotalLab software V 1.11 (Phoretix, Durham, NC).

Statistical Analysis

Group mean values were compared by two-tailed Student's *t*-test. Values of $P \leq 0.05$ were considered significant.

RESULTS

T Cell Factors are Strong Inducers of AP in Human BMSC and Dedifferentiated Human Osteoblasts (hOB)

To examine the effect of activated T cells on the differentiation of pre-osteoblasts to the osteoblast lineage, human BMSC were treated with 25% ACTTCM for 72 h then analyzed for AP activity, a specific marker of osteoblast lineage commitment (Fig. 1A). ACTTCM treatment resulted in a 3.4-fold increase in AP activity compared to control cultures. To determine the effect of activated ACTTCM on committed osteoblasts induced into a dedifferentiated state by subculture, cells were plated and allowed to attach overnight. Cell layers were treated with either medium alone, 25% un-activated T cell conditioned medium, or ACTTCM (12.5% or 25%) for 48 h then analyzed biochemically for AP. The results (Fig. 1B) show that at a concentration of 12.5% ACTTCM, AP was enhanced threefold, while 25% ACTTCM induced an approximately fourfold increase over control cells incubated in medium alone. AP levels in cells treated with un-activated T cell conditioned medium at 25% were no different than control cells incubated in medium alone.

T Cell Factors Rapidly Induce the Osteoblast Phenotype in Human BMSC

Human BMSC were treated with ACTTCM for 0–48 h and then RNA analyzed for the osteoblast specific factors Runx2 (a key osteoblast transcription factor) and OCN. As shown in Figure 2A, prior to treatment with ACTTCM, BMSC express only low basal levels of message

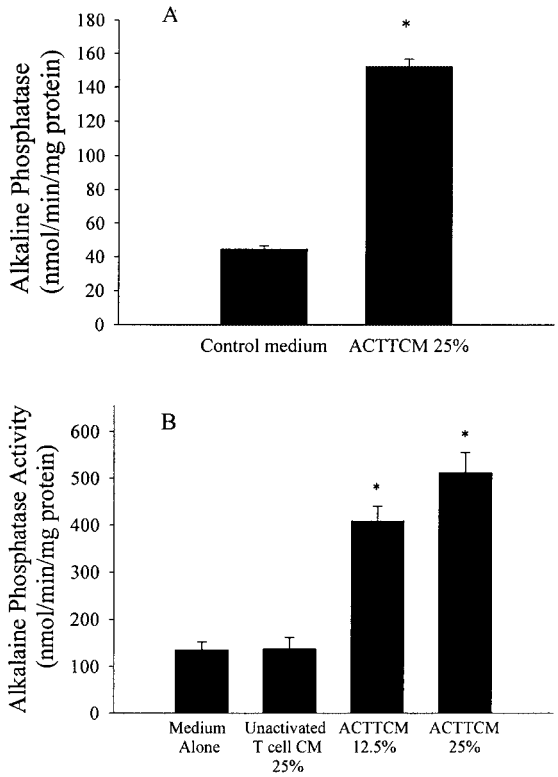

Fig. 1. ACTTCM rapidly enhances AP in BMSC and dedifferentiated hOB. **A**: BMSC were incubated with medium alone or 25% ACTTCM for 72 h. Cell layers were then analyzed biochemically for AP. Data are representative of the mean ± SEM (n = 4). $P < 0.001$ versus medium alone control. **B**: Subcultured hOB were allowed to attach overnight then incubated with medium alone, 25% un-activated T cell conditioned medium (T cell CM), or ACTTCM (12.5% or 25%) for 48 h. Cell layers were then analyzed biochemically for AP. Data are representative of the mean ± SEM (n = 8). *$P < 0.001$ versus medium alone control or un-activated T cell CM.

for Runx2. However, after 16 h of treatment, Runx2 message had increased fourfold and elevated to 10-fold by 24 h with maintenance through 48 h. OCN message in BMSC increased threefold by 24 h and sustained a maximum fourfold expression through 48 h (Fig. 2B). Together, these data demonstrate that T cell derived factors potently stimulate differentiation of BMSC into the osteoblast phenotype.

We next asked whether osteoblastic derived regulators of osteoclastogenesis were regulated by ACTTCM as well. Total RNA from BMSC was subjected to RT-PCR for receptor activator of NF-κB ligand (RANKL), receptor activator of NF-κB (RANK) and the orphan decoy receptor for RANKL, osteoprotegerin (OPG). We did not find expression of RANKL in BMSC at any time point tested (data not shown). However, surprisingly, RANK was strongly up-regulated in BMSC (Fig. 2C), increasing 5–6 fold over 24–48 h, respectively, and was temporally related to the expression of Runx2 expression. OPG expression was found to be unchanged between 0–24 h but increased marginally (~0.5 fold) at 48 h (Fig. 2D).

Since ACTTCM induced AP, an early marker of osteoblast differentiation, we examined dedifferentiated hOB cultures for expression of Runx2 to determine whether ACTTCM altered its expression. Figure 3A shows that Runx2 expression in the hOB was not altered over the entire time course tested suggesting that Runx2 was already maximally expressed in primary osteoblasts. We next examined the effect of ACTTCM on the induction of OCN, a later marker of osteoblast differentiation and activity (Fig. 3B). ACTTCM induced OCN approximately threefold by 8 h and maintained that level for 24 h before falling back to control levels at 48 h, suggesting that ACTTCM regulates osteoblast activity as well as differentiation.

T Cells Secrete Cytokines Which Modulate RANKL, RANK, OPG and TRAIL in Human Osteoblasts

Since activated T cells have been shown to stimulate osteoclastogenesis, we examined the effect of ACTTCM on the following osteoclast regulating factors: RANKL, RANK, OPG, and a receptor competitor for OPG, TRAIL, using semi-quantitative RT-PCR. As shown in Figure 4A, in contrast to BMSC where RANKL mRNA was undetected, hOB constitutively expresses RANKL mRNA. RANKL was found to be weakly up-regulated after treatment with 25% ACTTCM for 8 h and was sustained through 48 h. Although RANK was not constitutively expressed by hOB, after treatment with ACTTCM, RANK expression was potently induced by 8 h, maximally expressed by 16 h and sustained for 48 h (Fig. 4B). hOB constitutively expressed OPG mRNA (Fig. 4C) which was moderately up-regulated by ACTTCM within 8 h, maximally expressed by 16 h and was sustained for the 48 h incubation period. Since osteoblasts induce osteoclasts in cocultures in which both RANKL and OPG are induced [Yasuda et al., 1998b], we examined hOB for a receptor of OPG, TNF-related apoptosis-inducing ligand (TRAIL), to determine whether it may be up-regulated along with OPG (Fig. 4D). RT-PCR analysis demonstrated

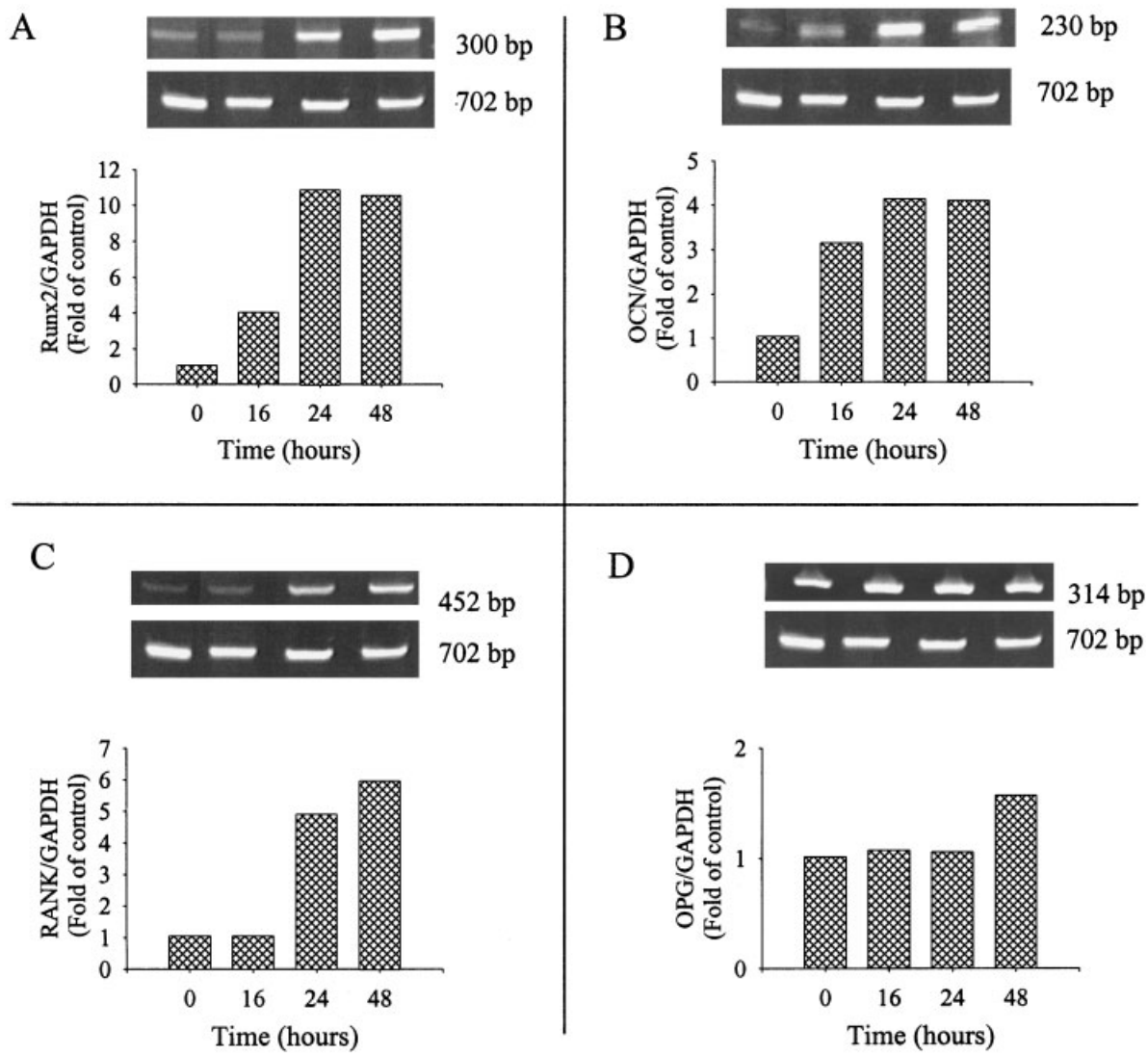

Fig. 2. ACTTCM rapidly induces the osteoblast phenotype in BMSC. BMSC were seeded into 100-mm dishes and grown to confluence. The medium was changed to MEM-α, 0.2% HIFBS and allowed to incubate for 24 h, before the addition of ACTTCM. Cells were then treated with 25% ACTTCM and total RNA was isolated after 16, 24, or 48 h incubation. Zero hour represents RNA isolated from cells prior to the addition of ACTTCM. Total RNA (2 μg) was used for RT-PCR using specific primers to (**A**) Runx2, (**B**) OCN, (**C**) RANK, and (**D**) OPG.

that TRAIL is constitutively expressed in hOB and that ACTTCM elevated expression approximately twofold over control within 8 h. This level of expression was maintained through 24 h then declined to approximately 1.5-fold of control at 48 h. Based on this data, we speculate that up-regulation of TRAIL may antagonize the elevated levels of OPG, thus mitigating its inhibitory effect on osteoclastogenesis.

DISCUSSION

We demonstrate in this study that exposure of BMSC to activated T cell derived factors results in osteoblast differentiation and activation leading to the enhanced expression of osteoclast regulatory factors. Although we do not pinpoint all of the T cell factor(s) that are responsible for the regulation of the osteoblast differentiation cascade, we do demonstrate that in nature, inflammation results in multiple interacting T cell derived cytokines capable of regulating osteoblastogenesis. Most importantly, the elevated bone remodeling that occurs in inflammatory diseases is complex and we demonstrate that T cells may play an essential role.

When osteoblasts are either initially isolated or subcultured and plated, the majority of the

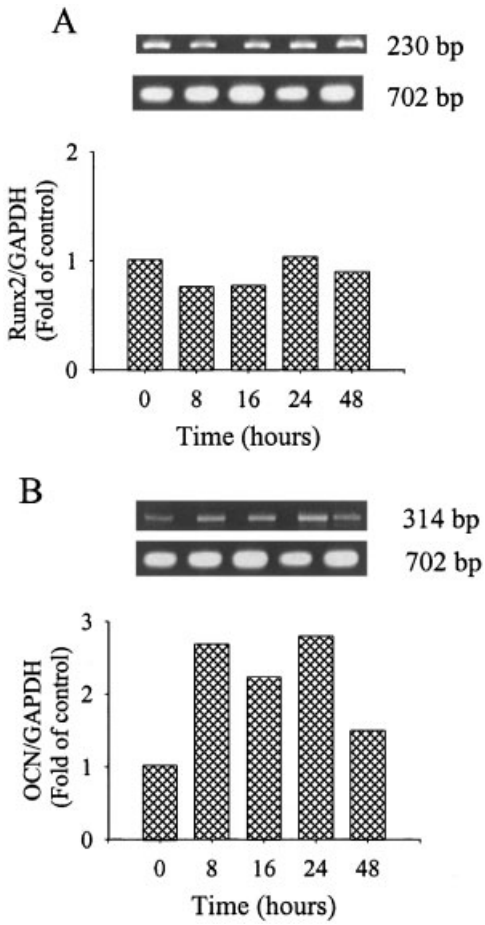

Fig. 3. Activated T cells regulate OCN, but not RUNX2, in hOB in a time dependent manner. hOB were cultured in 100 mm tissue culture dishes until confluent. The medium was changed to contain 0.2% HIFBS. After 24 h the medium was again changed and 25% ACTTCM added. RNA was harvested from cells at the noted time points. Zero hour represents RNA isolated from cells prior to the addition of ACTTCM. Total RNA (2 μg) was subjected to RT-PCR using specific primers to (**A**) Runx2 and (**B**) OCN.

osteoblastic phenotypic genes are down-regulated [Stein and Lian, 1993]. The major osteoblastic genes, such as AP and OCN, usually require a substantial period of time to re-express, usually 2 weeks or more [Stein et al., 1990]. Although other factors, such as estradiol, have been shown to increase AP in hOB cultures [Waters et al., 2001], the time frame for this regulation is much longer than the events reported in this study. Our data suggest that in vivo, the processes of activation of resting lining cells and differentiation of pre-osteoblasts committed to the osteoblast lineage may be rapidly stimulated by T cell factors to initiate both bone formation and bone resorption.

The primary factor that regulates osteoclastogenesis during normal bone remodeling, RANKL, is produced by osteoblasts and has been thoroughly examined with respect to the regulation of bone resorption [Lacey et al., 1998; Yasuda et al., 1998b]. T cells also produce RANKL [Wong et al., 1997; Weitzmann et al., 2001] which has been identified as a key factor in the osteopenia associated with RA [Kong et al., 1999]. RANKL binds to RANK, its receptor on pre-osteoclasts, and signals their differentiation into active osteoclasts [Li et al., 2000]. Of great interest was the finding that ACTTCM strongly induces the expression of RANK on both BMSC and hOB. Although the function of RANK on the hOB is not presently known, our data suggest that this receptor may play some yet to be determined role in pre-osteoblast differentiation and differentiated osteoblast function. Further studies will be needed to understand expression and function of RANK in cells of the osteoblast lineage.

Opposing RANKL is the soluble protein, OPG, produced by osteoblasts in response to Vitamin D3, PTH, and IL-11 [Yasuda et al., 1998a] as well as IL-1 and TNF-α [Hofbauer et al., 1998]. And opposing OPG is TRAIL, a tumor necrosis factor-related ligand that induces apoptosis upon binding to its death domain-containing receptors, DR4 and DR5 [Wiley et al., 1995; Walczak et al., 1997]. In the presence of TRAIL, OPG loses its ability to inhibit RANKL induced osteoclastogenesis [Emery et al., 1998]. Our studies showing that human osteoblasts express TRAIL confirm that of others [Alexander et al., 2001; Evdokiou et al., 2002]. And most importantly, TRAIL induces apoptosis in human osteogenic sarcoma cells, but not in normal human osteoblasts, despite their expression of the receptors for TRAIL [Evdokiou et al., 2002]. Furthermore, our finding that ACTTCM can induce the expression of TRAIL within the same time frame as that of RANKL and OPG highly suggests a role for this factor in the process of inflammation mediated osteopenia. In this regard, our data suggest that TRAIL may neutralize the effect of OPG leading to enhanced osteoclast formation via unchallenged RANKL. Of course we cannot ignore the fact that the effect of these cytokines is highly dependent on stoichiometry. Thus further studies will need to be performed to explore these possibilities.

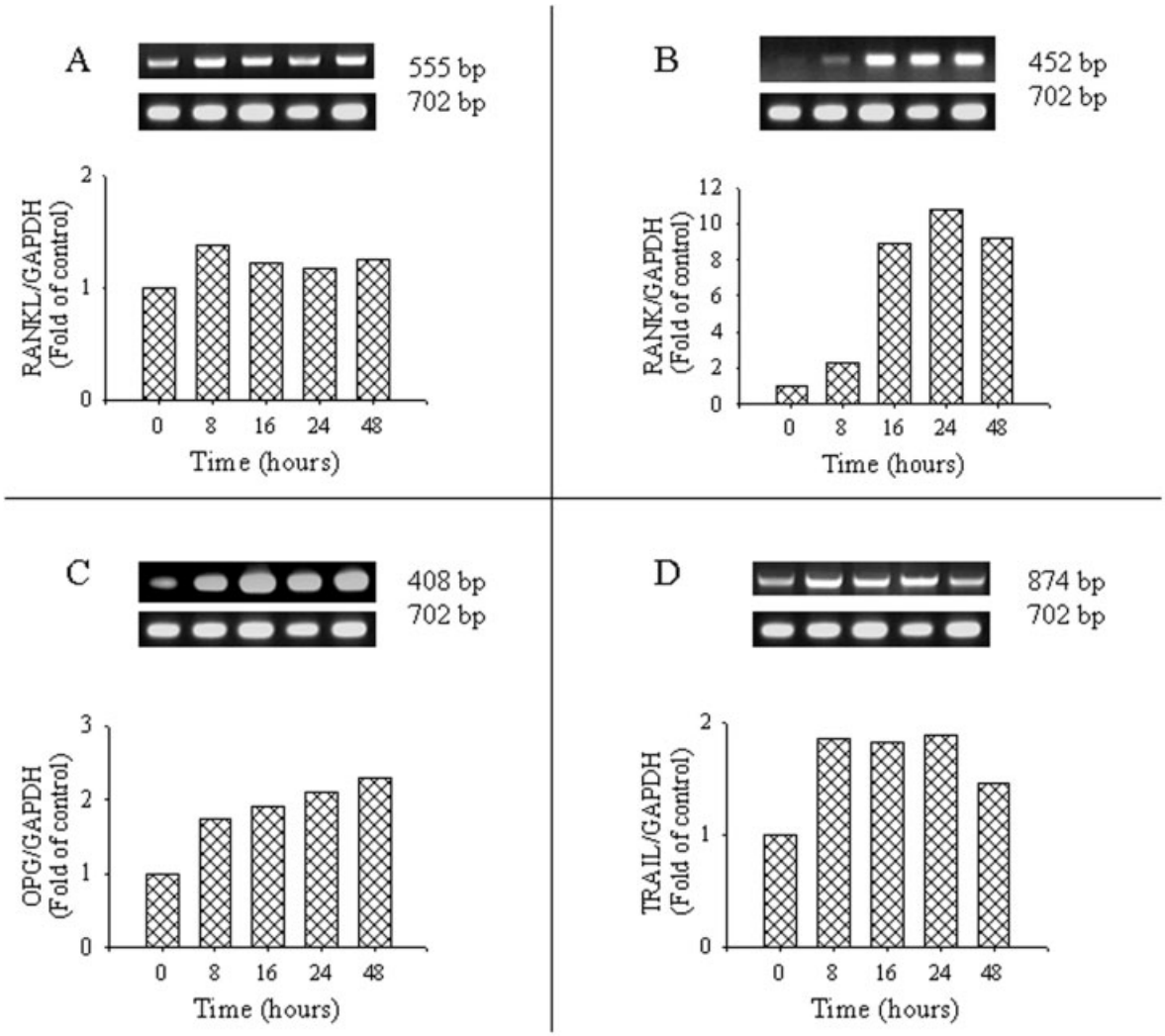

Fig. 4. ACTTCM modulates osteoclast regulatory factors in hOB in a time dependent manner. hOB were cultured in 100 mm tissue culture dishes until confluent. The medium was changed to contain 0.2% HIFBS. After 24 h the medium was again changed and 25% ACTTCM added. RNA was harvested from cells at the noted time points. Zero hour represents RNA isolated from cells prior to the addition of ACTTCM. Total RNA (2 μg) was subjected to RT-PCR using specific primers to (**A**) RANKL, (**B**) RANK, (**C**) OPG, and (**D**) TRAIL.

Our finding that ACTTCM has a powerful effect on pre-osteoblast differentiation to the osteoblast lineage is a highly novel observation. Our data suggest that the process of osteoblastogenesis may be driven even higher through the recruitment of the pre-osteoblast pool in the bone marrow. In fact, this possible recruitment may be responsible for the elevated osteoblastic activity associated with high bone turnover in RA. This scenario is supported by the evidence that patients with active disease have elevated circulating levels of both bone-specific AP and OCN [Gough et al., 1998], hallmarks of active osteoblasts [Suzuki et al., 1998]. Furthermore, our data demonstrating ACTTCM induction of rapid expression of both Runx2 (the master osteoblast transcription factor [Ducy et al., 1997]), and OCN (the most specific osteoblastic protein) are further evidence of such recruitment.

Of great interest was the absence of RANKL on highly purified human BMSC (extensively characterized as previously described [Cheng et al., 1994; Rifas et al., 1995], despite numerous studies citing the expression of RANKL on stromal cell lines and stromal elements of giant

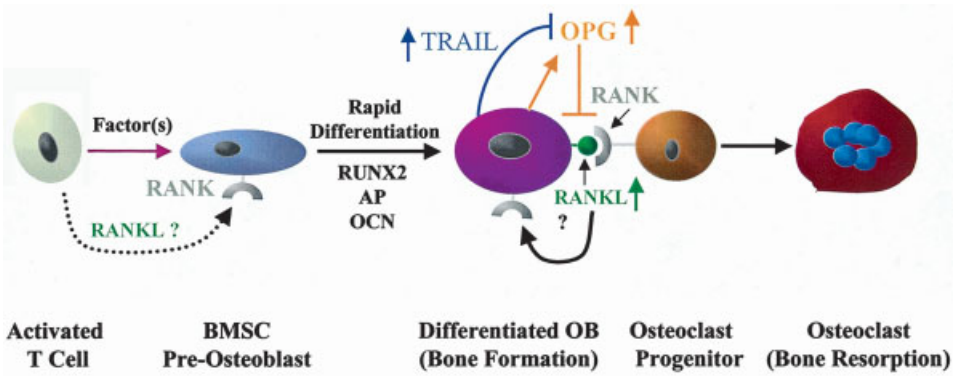

Fig. 5. Model depicting the role of activated T cells in the regulation of inflammation mediated bone resorption. Activated T cells produce a variety of cytokines which regulate the pre-osteoblast (BMSC) to differentiate into a mature, active osteoblast (OB). Upon stimulation a rapid process of differentiation is induced including the up-regulation of RUNX2 and OCN expression as well as AP activity. The differentiated osteoblast, under the stimulation of T cell factors, then would express multiple factors that regulate osteoclastogenesis. Receptor activator of NF-κB ligand (RANKL), the primary osteoclastogenic factor, would be up-regulated. But osteoprotegerin (OPG), which inhibits osteoclast formation, would also be up-regulated. However, TRAIL would be up-regulated as well. Since TRAIL inhibits OPG, this interaction may be a mechanism by which osteoclast formation would continue unimpeded. Furthermore, receptor activator of NF-κB ligand (RANK) would be up-regulated in the osteoblast and may act as an autocrine receptor for RANK ligand. The net result would be a balance favoring osteoblast differentiation and osteoclast formation and resorption. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cell tumors [Hicok et al., 1998; Atkins et al., 2000; Mancino et al., 2001; Tintut et al., 2002]. This data strongly suggest that in inflammatory diseases such as RA, a T cell–stromal cell interaction may be pivotal in initiating the processes of stromal cell differentiation into mature RANKL expressing osteoblasts, further amplifying osteoclastogenesis. Thus, based on these results, we propose a model (Fig. 5) for the role of T cell directed bone loss in chronic inflammation, as occurs in RA. Activated T cells produce a variety of cytokines which induce BMSC to differentiate into a phenotypic osteoblast by regulating the expression of Runx2, AP, and OCN. Consequently, the activated T cell drives bone loss via accelerated coupling of bone formation and bone resorption. On the other hand, OPG, which inhibits osteoclast formation, is also up-regulated, as is TRAIL. Since TRAIL in turn inhibits OPG, this interaction may be a mechanism by which osteoclast formation would continue unimpeded ensuring sustained support of osteoclastogenesis and the enhanced osteopenia associated with RA.

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