Perspective Article

Osteocyte biology: Its implications for osteoporosis

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Abstract

Osteocyte viability may play a significant role in the maintenance and integrity of bone. Bone loss due to osteoporosis may be due in part to osteocyte cell death. We have identified a factor that will protect both osteoblasts and osteocytes from cell death due to agents known to be responsible for various forms of osteoporosis. Not only does estrogen preserve osteoblast and osteocyte viability, but so does a molecule called CD40Ligand. This molecule is expressed on activated T lymphocytes, human dendritic cells, and human vascular endothelial cells, whereas its receptor CD40 is expressed on normal epithelium, B cells, and dendritic cells. CD40Ligand protects osteoblasts and the MLO-Y4 osteocyte-like cells against apoptosis induced by glucocorticoids, tumor necrosis factor α or etoposide. As tumor necrosis factor a has been shown to be responsible for post-menopausal bone loss and glucocorticoids induce dramatic bone loss, this finding has important implications with regards to potential therapy for both post-menopausal and steroid-induced osteoporosis.

Keywords: Osteocyte, Apoptosis, Bone Resorption, Osteoporosis, CD40Ligand

Introduction

Early hypotheses gave the osteocyte the function of responding to mechanical strain with the generation of biochemical signals that led to new bone formation. Later hypotheses stated that it was the lining cell that blocked the osteocyte from sending any signals under conditions of new bone formation[1]. The osteocyte may function to send signals for bone resorption. For example, accelerated bone resorption occurs with immobilization and with pathological overload[2]. The osteocyte subjected to immobilization is susceptible to hypoxia and nutrient deficiency whereas the osteocyte subjected to pathological overload is exposed to damaged bone in the form of microcracks and is therefore susceptible to damage to the cell body and dendrites. Bone loss occurs upon the menopause. Osteocytes in the absence of estrogen are less viable[3]. In all of these conditions, the viability of the osteocyte is compromised. This has led to the hypothesis that the dying or compromised osteocyte is responsible for send-

ing signals of bone resorption. Whether the signals are the same or different for these three conditions is still debatable.

Osteoclasts and osteoblasts are defined by function. Osteoclasts are defined as cells that resorb bone and osteoblasts are defined as cells that make bone. Osteocytes are defined by location, i.e. cells in a mineralized matrix. A lack of functional definition implies a lack of knowledge of function. It has been hypothesized that the major function of osteocytes is to translate signals related to mechanical strain into biochemical signals between osteocytes and cells on the bone surface. This is yet to be proved unequivocally.

To test the hypothesis that osteocytes can signal bone resorption, we have also shown that the MLO-Y4 cells are potent supporters of osteoclast formation and activation in the absence of 1,25, $(OH)_2$ vitamin D_3[4]. It was determined that these cells secrete large amounts of M-CSF and express large amounts of RANKLigand protein along their dendritic processes. As the dendritic processes of osteocytes have been shown to come into contact with cells in the marrow[5], this suggests that osteocytes may be able to support osteoclast formation via these processes. Recently we have also found that MLO-Y4 cells will support the proliferation and chemotaxis of two osteoclast precursor cell lines, MOCP-5 and RAW 264.7. MLO-Y4 cells may represent exposed osteocytes outside the protection of the mineralized matrix or may represent immobilized osteocytes compromised due to a lack of mechanical strain. Future experiments include

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treating these cells with estrogen and CD40L to determine effects on their ability to support osteoclast activity.

The CD40-CD40 Ligand (CD40L) signaling system clearly plays an important role in immune cell death and function, however little is known concerning the role of this signaling system in bone. CD40L is expressed on activated T lymphocytes, human dendritic cells, and human vascular endothelial cells. CD40 is 50 Kd glycoprotein expressed not only on the surface of B cells and dendritic cells, but also on normal epithelium and some epithelial carcinomas[6]. The receptor signals by binding to members of TNF receptor associated proteins (TRAF) such as TRAF 2 and TRAF 6 appear to activate NFkB transcription factor. Osteocytes and osteoblasts express CD40 on their surface and CD40L clearly maintains viability in these bone cells[7].

Materials and methods

Reagents for Apoptosis staining included Propidium Iodide (Sigma, St Louis,MO) and Annexin V FITC (Pharmingen, San Diego, CA). Soluble CD40L (trimeric form) was provided by Immunex (Seattle, WA). Tissue culture media DMEM and Fetal bovine serum was obtained from Life technologies (Grand Island, NY) and Calf serum (CS) was from Hyclone Laboratories, Inc (Logan, UT). Recombinant TNFα was obtained from R and D (Minneapolis, MN). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Treatment

The MLO-Y4 cells were pretreated with CD40L (0.5-1.5 μg/ml) for 15 minutes followed by treatment with either dexamethasone at 10^{-6}M, TNFα at 1 ng/ml or etoposide at 50 μM for 6 hrs. The MLO-Y4 cells were cultured in 2.5% FBS/2.5%CS, 5% CO_2, 37ºC.

Assays for cell death/Apoptosis

Below are three different assays for the determination of cell viability, trypan blue exclusion, nuclear morphology and FACS staining for Annexin V/Propidium iodide. All assays were performed as described previously[7]. Briefly, for trypan blue exclusion the cells were stained and the percentage of trypan blue positive cells in each culture condition was used to calculate cell survival[8]. Nonadherent cells were combined with adherent cells released from the culture dish using trypsin-EDTA, resuspended in medium containing serum, and collected by centrifugation. Subsequently, 0.04% trypan blue was added and the percentage of cells exhibiting both nuclear and cytoplasmic staining was determined using a hemocytometer. At least 100 cells per condition were counted. For nuclear morphology, MLO-Y4 cells were used that were stably transduced with retroviral vector carrying the green fluorescent protein (GFP) cDNA with a nuclear localization sequence designed to target the GFP to the nucleus.

The cells were fixed in neutral buffer formalin for 8 min, and apoptosis was assessed by enumerating cells exhibiting chromatin condensation and nuclear fragmentation under a fluorescent microscope. At least 500 cells from fields selected by systematic random sampling were examined for each experimental condition[3,9]. For FACS staining for Annexin V/Propidium iodide the single cell suspensions were washed twice with PBS and resuspension in 1X binding buffer (0.01 M HEPES/NaOH, pH 7.4; 140 mM NaCl; 2.5 mM $CaCl_2$) at a concentration of 1 x 106 cells/ml. The cells were aliquoted (100 μl) into polypropylene tubes and incubated with Annexin V FITC (5 ml) and 50 μg/ml of Propidium Iodide (10 ml) for 15 minutes at 4ºC in the dark and analyzed by flow cytometry within 1 hr. Flow cytometric analysis was performed on a FACSCalibur using CELLQuest software (Becton Dickinson, Mountain View, CA) by analyzing 5-10 x 10^4 cells /sample using forward and side scatter gates to include late apoptotic cells.

Results

CD40L prevents glucocorticoid and TNFa induced apoptosis in MLO-Y4 osteocytes.

MLO-Y4 cells have several characteristics of primary osteocytes[10] including numerous dendritic processes, express low or no collagen type I and alkaline phosphatase and express high amounts of osteocalcin. This cell line does not express an antigen specifically found on early osteoblast progenitors known as osteoblast specific factor 2 (OSF-2) or more recently as "periostin"[11,12]. These cells also express large amounts of the osteocyte-specific antigen, E11[13], an antigen shown to localize to only osteocytes *in vivo* as described by Schulze and coworkers[14].

Trypan blue exclusion, nuclear morphology, and Annexin V staining were used as a measure of cell death in MLO-Y4 cells pretreated with CD40L followed by dexamethasone, TNFa, or etoposide. The percentage of apoptotic cells was significantly lower in cells that had been pretreated with CD40L independent of the proapoptotic agent used. Consistent with these results, cultures of MLO-Y4 cells stably transfected with nuclear green fluorescent protein pretreated with CD40L contained a significantly lower percentage of cells exhibiting nuclear fragmentation and/or chromatin condensation after treatment with dexamethasone, TNFα, or etoposide. Similar to the results obtained with the above two methods, the percentage of apoptotic cells identified by Annexin V staining was lower in the cells pretreated with CD40L followed by dexamethasone or TNFα. The data from these different apoptosis assays are summarized in Table 1.

Discussion

The most common form of osteoporosis is postmenopausal, due to a lack of estrogen. Estrogen has been

ASSAY	TRYPAN-BLUE	GFP	ANNEXIN V
CD40L	1.26	0.77	1.07
Dex	2.40*	2.12#	2.21*
Dex + CD40L	1.37	0.88##	1.28
TNF	2.70*	1.55#	1.70*
TNF + CD40L	2.26	1.33#	1.30
Etoposide	2.77*	1.41#	N.D.
Etoposide + CD40L	1.39	0.74##	N.D.

Table 1. CD40Ligand significantly reduces apoptosis due to glucocorticoid, tumor necrosis factor α, and etoposide in the MLO-Y4 cells. The cells were pretreated with CD40L (0.5 µg/ml) for 15 minutes followed by incubation with dexamethasone (10-6M), TNFα (1 nM) or etoposide (50 µM) for 6 hours. The cells were then analyzed for cell survival by trypan blue exclusion assay, apoptosis by nuclear morphology, and the Annexin V staining assay as described in the methods. The results are expressed as treated to control ratios. Control is medium alone. *, Annexin V and trypan-blue, significantly different from control using one way ANOVA, $p<0.05$. #, for GFP, significantly different from controls, $p<0.07$; ##, significantly different from the pro-apoptotic agent alone using chi square test, $p<0.025$. N.D.= not determined.

shown to be a viability factor or anti-apoptotic factor for osteocytes and osteoblasts in both humans and rodents[15,16]. Cytokines such as Tumor Necrosis Factor α and Interleukin-1 have been reported to increase with estrogen deficiency[17,18]. Delivery of both the soluble TNF receptor and an IL-1 receptor antagonist completely blocked bone loss due to ovariectomy in mice[19].

An increase in osteoblast/osteocyte apoptosis has also been demonstrated in patients with glucocorticoid-induced osteoporosis[20]. Apoptosis of osteocytes may play an important role in this third most common cause of osteoporosis[21]. Mice administered glucocorticoids show higher numbers of apoptotic/dead osteoblasts that appear to be responsible for the decreased bone formation[8]. In addition, these studies demonstrated an increase in apoptotic osteocytes that may contribute to bone fragility independent of changes in bone mass[3,8].

CD40L clearly prevented apoptosis due to three very different agents, a steroid (dexamethasone), a cytokine (Tumor Necrosis Factor α) and an apoptosis-inducing chemical compound (etoposide). All three are purported to work by different pathways through different receptors to induce apoptosis. The CD40 receptor signals by binding to members of TNF receptor associated proteins (TRAF) such as TRAF 2 and TRAF 6 that appear to activate NFkB transcription factor. This pathway may be responsible for interfering with the other three pathways. Therefore, CD40L may support viability of osteocytes against a wide array or variety of apoptotic factors independent of signaling or transcriptional mechanisms.

CD40L induces apoptosis in cancer cells but has the opposite effect on normal dendritic cells acting as an anti-apoptotic factor[22]. CD40L is a promising anti-cancer agent because of this property. Thus, this receptor/ligand signaling pathway may promote cell survival or cell death not only depending on cell type or the stage of differentiation but also on state of transformation. As this agent is already being used in clinical trials, toxicity or side effects should not be an issue in osteoporosis prevention trials.

The commitment to undergo cell death can be influenced by a number of intracellular and extracellular events. In multicellular organisms, all cells are programmed to commit suicide if survival signals are not received from their environment. These survival signals can be provided by the neighboring cells, by extracellular matrix, or by growth factors[23-28]. It will be important to determine the source of CD40L in the bone microenvironment.

Factors that initiate apoptosis in one cell type may block apoptosis in another cell type. For example, estrogen prevents apoptosis of osteoblastic cells, but induces apoptosis of osteoclasts[29,30]. Interleukin-1 and Tumor Necrosis Factor a are apoptotic factors for osteoblasts, but anti-apoptotic factors for osteoclasts. It will be important to determine the effect of CD40L on osteoclast formation and function.

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