A role of TRAIL in killing osteoblasts by myeloma cells

Inge Tinhofer,[†,‡,1] **Rainer Biedermann,**[#] **Martin Krismer,**[#] **Roman Crazzolara,**[§,‡] **and Richard Greil**[†,‡]

[†]Laboratory of Immunological and Molecular Cancer Research (LIMCR), 3rd Medical Department of the Salzburg General Hospital and Private Paracelsus Medical University, Salzburg; [‡]Tyrolean Cancer Research Institute at the University of Innsbruck; #Department of Orthopedic Surgery; [§]Department of Pediatrics, University Hospital Innsbruck, Innsbruck, Austria

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SPECIFIC AIMS

Tumor necrosis-related apoptosis-inducing ligand (TRAIL), a member of death receptor ligands, is a vital component of immunosurveillance of tumor cells by both CD8$^+$ T cells and NK cells. Its importance in tumor control as well as its safety profile toward normal tissues raised great hopes of an encouraging outcome from early clinical trials. Besides tumor control by TRAIL$^+$ immune cells, there is accumulating evidence that death receptor ligands might also be used by tumor cells themselves to kill bystander cells, a mechanism called "the tumor counterattack." Studies from our group revealed that myeloma cells expressed functional Fas ligand (FasL) by which they could kill a Fas-sensitive T cell line in vitro. Erythroblasts were identified as alternate targets for FasL- and/or TRAIL-mediated suppression by myeloma cells. In the present study, we tried to elucidate whether the formation of bone lesions that develop with high frequency in myeloma patients is mediated at least in part by interaction of myeloma cells and osteoblasts through FasL and/or TRAIL, thereby inducing cell death in the bone cell fraction.

PRINCIPAL FINDINGS

1. Osteoblasts express both Fas and TRAIL-R, but are only sensitive to Fas-induced apoptosis

After establishing primary osteoblasts from trabecular bone fragments (n=15), we determined whether these cells express the essential constituents of death receptor signaling, namely, Fas and TRAIL-R. We found high expression of Fas/CD95 and moderate expression of TRAIL-R2/DR5, but failed to detect TRAIL-R1/DR4. While expression of Fas rendered these cells sensitive to apoptosis as detected by an increase in phosphatidylserine exposure after incubation with recombinant soluble FasL, the addition of recombinant human TRAIL had only a minor cytotoxic effect in osteoblasts.

2. Killing of osteoblasts by myeloma cells is partly mediated by TRAIL

We first addressed the question of whether osteoblasts are targets for death receptor ligand-mediated killing by myeloma cells. Osteoblasts were labeled with the cell-permeant cell tracker CFSE and cocultured for 72 h at a target:effector ratio of 1:5. In the presence of myeloma cells, the percentages of apoptotic osteoblasts increased significantly (**Fig. 1**). Preincubation of RPMI-8226 cells with antagonistic anti-FasL antibody NOK-1 was ineffective in blocking killing of osteoblasts whereas the addition of FcDR5 significantly reduced the percentage of apoptotic osteoblasts (Fig. 1). Similar results were seen when LP-1 or OPM-2 cells were used as effector cells (Fig. 1 and data not shown).

3. Killing of osteoblasts is mainly mediated by a membrane-bound factor(s)

To determine whether the cytotoxic activity of myeloma cells is mediated by a soluble factor or is dependent on direct contact between target and effector cells, we separated osteoblasts and myeloma cells during coculture experiments using transwell cell culture inserts with 0.4 μm pore size. Comparison of myeloma-mediated cytotoxicity after direct or transwell coculture suggested that primarily membrane-bound factors are involved.

4. Increased sensitivity of osteoblasts to recombinant TRAIL in coculture with myeloma cells

Since we observed TRAIL-mediated cytotoxicity toward osteoblasts in coculture experiments with myeloma cells, we next addressed whether recombinant TRAIL would further increase cell death of osteoblasts when

[1] Correspondence: Laboratory of Immunological and Molecular Cancer Research, 3rd Medical Department of Hematology, Oncology, Hemostaseology, Rheumatology and Infectiology, University Hospital Salzburg, Austria. E-mail: i.tinhofer@salk.at

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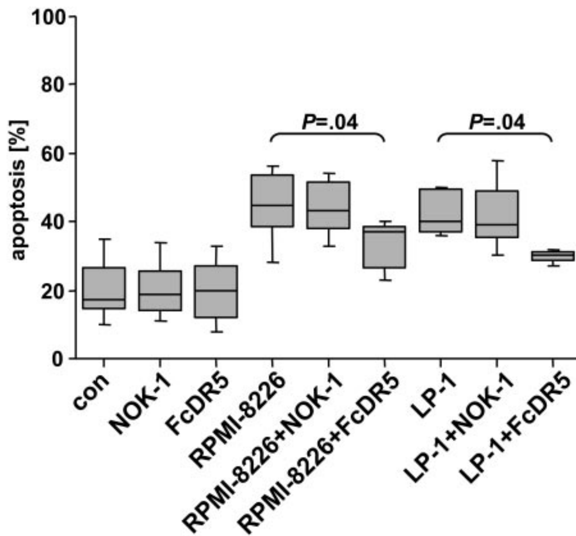

Figure 1. Myeloma cells kill primary osteoblasts in a partly TRAIL-dependent pathway. RPMI-8226 or LP-1 cells left untreated or pretreated for 1 h with the specific TRAIL inhibitor FcDR5 (0.5 μg/mL) or the antagonistic anti-FasL mab NOK-1 (5 μg/mL). Subsequently, myeloma cells were washed and cocultured with CFSE-labeled primary osteoblasts at a ratio of 5:1 for 72 h. Apoptosis of osteoblasts was determined by flow cytometry using the annexinV-Alexa647/PI binding assay. Results from 5 independent experiments are presented.

cocultured with myeloma cells. Osteoblasts were either cultured alone or in the presence of RPMI-8226 or LP-1 cells for 24 h, followed by addition of recombinant TRAIL (50 ng/mL) for another 24 h. Subsequently, percentages of apoptotic osteoblasts were determined by the annexinV/PI assay. Osteoblasts were resistant toward TRAIL when cultured alone, while in coculture with RPMI-8226 or LP-1 cells a higher percentage of apoptotic osteoblasts was observed, which also increased after treatment with recombinant TRAIL (**Fig. 2**). On the other hand, RPMI-8226 or LP-1 cells were sensitive to treatment with recombinant TRAIL. Their sensitivity, however, decreased when they were treated in coculture with osteoblasts. The protective effect of osteoblasts on TRAIL-induced apoptosis of myeloma cells was partly mediated by their production of osteoprotegerin.

5. Native tumor cells from myeloma patients induce apoptosis in osteoblasts

To address the question of whether molecular interactions characterized in the myeloma cell line model in vitro also occur between myeloma cells and osteoblasts in vivo, we cocultured osteoblasts for 72 h with purified CD138+ myeloma cells from untreated myeloma patients (n=5). Primary myeloma cells significantly increased the percentage of apoptotic osteoblasts, which was blocked by preincubation of myeloma cells with FcDR5.

CONCLUSION

Skeletal complications such as lytic bone lesions, severe bone pain, pathological fractures, and hypercalcemia are often observed in patients with multiple myeloma. The underlying mechanism responsible for bone lesions in these patients is increased activity of osteoclasts, the bone-resorbing cells. Osteoclast-activating factors have been analyzed extensively, and several candidate proteins have been identified: receptor activator of nuclear factor-kappaB ligand (RANKL), MIP1a, IL-1β, IL-6, and tumor necrosis factor-α. Recently, gene expression profiling of myeloma cells from patients with or without lytic bone disease revealed no relation between the presence of bone disease and expression of osteoclast-activating factors such as RANKL, RANK, osteoprotegerin (OPG), $MIP1_{\alpha}$, PTHrP, and IL1 in myeloma cells. These data suggest that more indirect mechanisms involving the tumor microenvironment contribute to myeloma cell-induced bone disease.

In normal bone remodeling, osteoclasts resorb old or damaged bone, leaving space for osteoblasts to form new bone. Disturbances of this balance in patients with multiple myeloma are further characterized by a marked impairment of bone formation by osteoblasts. Suppression of osteoblasts in myeloma have also been demonstrated by Hjorth-Hansen and co-workers. They used an in vivo model of human myeloma producing bone disease in irradiated severe combined immunodeficiency disease mice and observed the development of a dramatic osteoblastopenia (i.e., a 99% reduction in

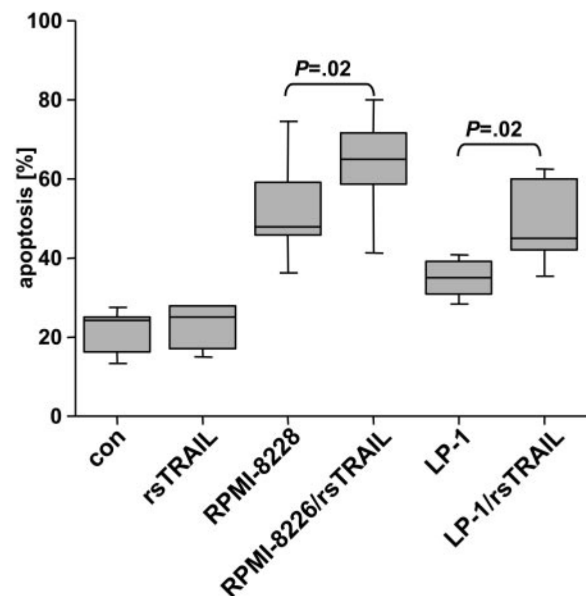

Figure 2. Primary osteoblasts in coculture with myeloma cells are sensitized to TRAIL-induced apoptosis. Primary osteoblasts were cocultured with RPMI-8226 or LP-1 cells for 72 h. For the last 24 h, TRAIL (50 ng/mL) was added to the culture. Subsequently, osteoblast apoptosis was determined by flow cytometry using the annexinV/PI binding assay. Results from 7 independent experiments are presented.

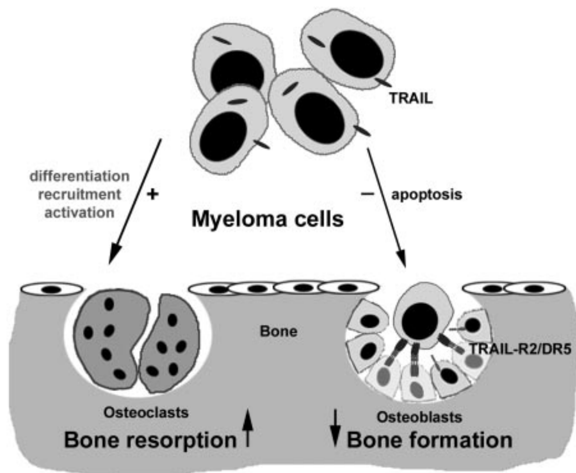

Figure 3. Schematic diagram of aberrant bone remodeling in multiple myeloma. Tumor cells express TRAIL and trigger TRAIL-mediated apoptosis in TRAIL-R2/DR-5+ osteoblasts. In addition, they stimulate recruitment and differentiation of osteoclast precursors as well as their activity as mature osteoclasts. Interactions of myeloma cells with bone-forming (osteoblasts) and bone-degrading cells (osteoclasts) might contribute to bone destruction in multiple myeloma.

osteoblast counts). The nature of inhibitors of osteoblast viability and activity in myeloma, however, has largely remained unclear, although a role for cytokines and death receptor ligands has been postulated recently.

An essential role of FasL in bone remodeling has been proposed by Katavic and co-workers, who found increased bone mass in *gld* mice known to have a functional defect in the FasL gene. The increase in bone mass resulted from enhanced bone-forming capacity of osteoblasts and correlated with reduced numbers of apoptotic bone-forming cells detected within the bone, strongly underlining a role of FasL in controlling osteoblast turnover. Our present study confirms previous reports showing that osteoblasts express Fas (CD95, APO-1) and the death-transducing member of the TRAIL receptors family, namely, TRAIL-R2. In agreement with the latter report, our results demonstrate that primary osteoblasts are insensitive to recombinant TRAIL while they undergo cell death after stimulation with agonistic anti-Fas mab. We first hypothesized that, if myeloma cells kill bone cells, they might do so via FasL but not via TRAIL. However, in coculture with myeloma cells that expressed FasL and TRAIL, we observed significant killing of osteoblasts, which was independent of FasL but partly dependent on TRAIL (Fig. 1). Of clinical importance, myeloma cell-induced killing of osteoblasts was not limited to myeloma cell lines but could also be observed when primary myeloma cells were used as effector cells.

Expression of TRAIL in myeloma cells is not part of the neoplastic transformation process, as TRAIL expression has also been demonstrated in normal IL-6-differentiated plasma cells. The presence of TRAIL on the surface of myeloma cells might be deleterious for tumor cell survival in view of their sensitivity to TRAIL-induced cell death observed in vitro. This lethal potential of TRAIL, however, should be counteracted in vivo by constitutive activation of NFκB signaling pathways in the neoplastic clone triggered by their interaction with bone marrow stromal cells. Myeloma cell lines cultured in vitro lacking supportive stimuli from their environment might protect themselves from TRAIL by downregulating their surface expression, but retaining an intracellular store that can be released at target cell contact. This might explain our observation that blocking of TRAIL by preincubation of cells with recombinant FcDR5 only partially blocked TRAIL signaling (Fig. 1).

All myeloma cell lines used here were sensitive to recombinant TRAIL. These data together with results from other groups suggest a potential therapeutic application of TRAIL in multiple myeloma. The concept of death receptor ligands in tumor therapy evolved after FasL and TRAIL were identified as endogenyous cytotoxic weapons of immune effector cells and the detection of their specific receptors on neoplastic cells. However, the fatal outcome of therapies targeting Fas, leading to severe damage of the liver and to death of treated animals, stopped the preclinical development of FasL as a therapeutic tool for tumor therapy. A new hope for the applicability of death receptor ligands in tumor therapy arose from observations that TRAIL, another member of the TNF family, selectively killed tumor cells and was nontoxic to the majority to normal cell types. The results of our study now show that normal tissues might be sensitized to the cytotoxic action of TRAIL by the neoplastic clone itself. In our experiments we used primary osteoblasts from healthy donors. Their short cocultivation with myeloma cells rendered them sensitive to TRAIL's cytotoxic action. This cytotoxicity could not be explained by the fact that we used a tagged version of TRAIL, because this version failed to kill normal osteoblasts when they were cultured alone. Instead, sensitivity of osteoblasts to TRAIL signaling was found to increase in the presence of myeloma cells whereas myeloma cells were less sensitive to recombinant TRAIL in coculture with osteoblasts. Thus, the efficacy of TRAIL in a therapeutic setting might be reduced in myeloma patients by a protective factor produced by osteoblasts. Osteoprotegerin should fulfill all criteria of such a factor, as it has been shown to be produced by osteoblasts and stromal cells, and to block TRAIL-induced apoptosis in myeloma cells. Addition of recombinant OPGL, which represents the high-affinity ligand of OPG and is able to decrease binding of OPG to TRAIL, could revert the protective effect of osteoblasts on TRAIL-induced apoptosis in myeloma.

In conclusion, the results of our study revealed a role for TRAIL in inhibiting bone formation in multiple myeloma. The identification of molecular mechanisms involved in sensitization of osteoblasts and desensitization of myeloma cells to TRAIL signaling might be a prerequisite for safety and efficacy of TRAIL in myeloma therapy. FJ