

TRAIL effect on osteoclast formation in physiological and pathological conditions

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1. ABSTRACT

Although osteoclasts (OCs) differentiate under the control of RANK/RANKL/OPG system, a number of inflammatory cytokines can contribute to increase osteoclastogenesis in diseases associated with bone loss. Recently, different studies indicate that TRAIL is implicated in modulating osteoclastogenesis. Here, we investigated the effect of TRAIL on OC formation in physiological and pathological conditions with bone involvement utilizing osteoclastogenesis *in vitro* models represented by peripheral blood mononuclear cells (PBMCs) from healthy donors and patients affected by multiple myeloma or periodontal disease. We demonstrated that in PBMCs from healthy donors TRAIL can directly induce OC formation in the absence of RANKL, while exert an inhibitory effect when added concomitantly to RANKL. In PBMCs from the patients, in which media the levels of TRAIL, RANKL and OPG are elevated, the neutralization of TRAIL partially inhibits the OC formation, and this effect was reversed by RANKL addition. Finally, we detect high TRAIL levels in the sera from the patients. In conclusion, our results indicate that TRAIL could exert a different role in modulating OC differentiation in physiological and pathological conditions.

2. INTRODUCTION

Osteoclasts (OCs), responsible for bone resorption, are multinucleated cells formed by the fusion of circulating hematopoietic precursor cells of the monocyte/macrophage lineage (1). It is well established that in physiological conditions OC differentiation is finely controlled by macrophage colony stimulating factor (M-CSF) and receptor activator factor of nuclear factor kappaB ligand (RANKL) (1). However, in pathological conditions a number of other cytokines and growth factors are known to impact OC formation and function either directly, by acting on cells of osteoclast-lineage, or indirectly, by acting on other cell types to modulate expression of the key osteoclastogenic factor, RANKL, and/or its inhibitor, osteoprotegerin (OPG) (2-6). Interestingly, recent studies demonstrated that several TNF family molecules, such as TNF-alpha or FasL, which is mainly known for its apoptotic role, can promote OC differentiation (7-9). Additionally, another molecule of TNF superfamily of cytokines, the tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL), well characterized as inducer of apoptosis in different tumor and normal cells, has been recently implicated in modulating the differentiation of hematopoietic progenitors towards

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different lineages, including OCs (10-12). However, contradictory findings have been provided from the literature on TRAIL involvement in osteoclastogenesis process. In particular, by using human peripheral blood mononuclear cells (PBMCs) and RAW264.7 murine monocyte cell lines as models of osteoclastogenesis, Zauli *et al.* demonstrated that TRAIL significantly inhibited MCSF- and RANKL-induced OC formation (12). On the contrary Yen *et al.* established that TRAIL induced monocyte/macrophage precursor cells to differentiate into active OCs in the same two models (13), and Labrinidis *et al.* found that TRAIL did not affect OC differentiation or bone resorption (14). With a completely different experimental approach, consisting in a co-culture model between mouse osteoblasts and RAW264.7, Nicolin *et al.* showed that only high TRAIL concentrations inhibited OC differentiation (15).

Moreover, contradictory data have been also published about the role of TRAIL on bone in *in vivo* animal models. Zauli *et al.* demonstrated that mice treated with recombinant TRAIL showed an increase in total bone mass (16). In contrast, Labrinidis *et al.* did not find changes in any bone histomorphometric parameters in TRAIL-treated animals (14). Although all these findings are clearly conflicting about the role of TRAIL on OC formation, different works agree on the apoptotic role of TRAIL on completely differentiated OCs (17-18). It is known that TRAIL engages its death receptors DR4 or DR5 to induce cell apoptosis, while TRAIL binding to “decoy” receptors, DcR1, DcR2 or OPG, fails to induce cell death (19). As we recently demonstrated, the sensitivity of OCs and their precursors to the apoptotic effect of TRAIL is critically regulated by the ratio between TRAIL death and decoy receptors, which are diversely expressed on cell surface during the different phases of OC differentiation (18). Intriguingly, we showed that only fully differentiated human OCs, which highly express the death receptor DR5, were susceptible to TRAIL-induced apoptosis, on the contrary OC precursors, as a result of their over-expression of the decoy receptor DcR2, were protected against TRAIL apoptotic effect (18). Finally, a crucial point that needs to be kept in mind is the interaction between TRAIL and OPG, which leads to the mutual neutralization of the two molecules as extensively demonstrated especially in pathological conditions (20-22). In particular, it has been demonstrated that OPG can function as a survival factor for different cell types which are sensitive to TRAIL-induced apoptosis (20-26). Additionally, the simultaneous presence of TRAIL and OPG leads to the loss of ability by OPG to inhibit OC formation, thereby TRAIL could function as osteoclastogenic factor. This is the case of multiple myeloma (MM), a hematological malignancy often associated with osteolytic lesions, in which OPG counteracts the apoptotic role of TRAIL (22). As we previously demonstrated, TRAIL through the interaction with OPG contributed to the elevated formation of long lifespan OCs from PBMCs of MM patients with osteolysis (22). Additionally we also showed elevated levels of TRAIL in the sera of periodontal disease (Pd) patients (27) and elevated osteoclastogenesis in PBMC cultures from the same patients, in which the loss alveolar bone occurs (28).

Thus, the role of TRAIL on osteoclastogenesis could be much more complex than it appears from the data currently present in the literature, and may be different in physiological or pathological conditions. Thus, in this study we investigated the role of TRAIL in the formation of OCs firstly in human PBMCs from healthy subjects, secondly in PBMCs from patients with MM bone disease and Pd.

3. MATERIALS AND METHODS

3.1 Patients

The samples included peripheral blood (PB) from 32 MM patients at diagnosis (15 males and 17 females, median age 65.5 +/- 8.7) and 30 periodontal disease (Pd) patients (25 males and 5 females, aged from median age 41.5 +/- 4.5). Control subjects were divided in two subgroups to match them for age and sex with MM patients (group 1) or Pd patients (group 2). The diagnosis of MM was established according to the International Myeloma Working Group (IMWG) (29) criteria and the staging was evaluated by the International Staging System (ISS) (30) for MM. In MM patients the presence of osteolysis was documented by skeleton standard radiography and in some cases also by nuclear magnetic resonance (NMR). In Pd patients the clinical periodontal evaluation was based on the Plaque Index (PI) (30), Gingival Index (GI) (31), and radiographic and clinical evidence of alveolar bone loss. Alveolar bone loss determination was performed using radiographic and clinical evidence concerning the distance between the cemento enamel junction (CEJ) and the level in which periodontal ligament appeared normal (32-33). Informed consent was obtained in keeping with institutional policies. Approval was obtained from the Institutional Review Board of the Departments of Periodontology and of Internal Medicine and Public Medicine of the University of Bari.

3.2 Cell cultures

OCs were obtained from PBMCs of control subjects and patients with Pd or MM bone disease. PBMCs were isolated by centrifugation over a Histopaque 1,077 density gradient (Sigma Chemical Co., St. Louis, MO, USA), plated at 1×10^6 cells/cm² in alpha-Minimal Essential Medium (alpha-MEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco Limited, Uxbridge, UK). To examine the effect of TRAIL on OC formation from PBMCs of subjects belonging to the controls of group 2, the cells were cultured for 18-21 days in the presence of 25 ng/ml recombinant human Macrophage Colony Stimulating Factor (rh-MCSF- R&D Systems Inc MN, USA) and increasing concentration rh-TRAIL (range 10-200 ng/ml - R&D Systems Inc). In parallel, PBMCs were cultured in the presence of MCSF, RANKL and 100 ng/ml TRAIL. As control PBMCs were cultured in the presence or in the absence of 25 ng/ml MCSF and 30 ng/ml receptor activator of the nuclear factor-kappa B ligand (rh-RANKL - R&D Systems Inc).

To examine the resorbing activity PBMCs were plated on multiwell slides coated with a calcium phosphate film (Millennium Biologix, Kingston, ON, Canada) in the

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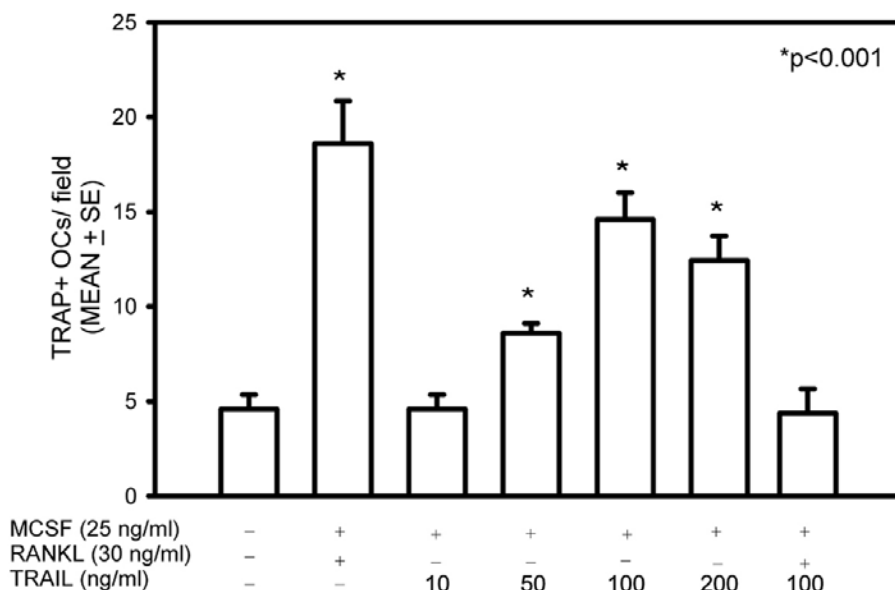


Figure 1. TRAIL induces osteoclast differentiation from PBMCs of healthy subjects. Adherent PBMCs seeded in a 96-well plate, were cultured with M-CSF in the presence of RANKL, increasing concentrations of TRAIL, or TRAIL and RANKL simultaneously. After 21 days, the cells were analyzed for osteoclastic differentiation. Multinucleated (more than 3 nuclei per cell) and TRAP+ cells were identified as OCs, and the number of cells/field was reported in graph as mean \pm SE. The graph shows a representative experiment in which each treatment has performed in quadruplicate.

same conditions of OCs differentiation experiments. To visualize the pits formed by the OCs, the cells were removed by adding NaOCl to each well. The photomicrographs of Figures 2 were obtained using a Nikon Ellipse E400 microscope equipped with Nikon Plan Fluor 10x/0.30 diel. The microscope was connected with a Nikon digital camera D_xM 1200. For some experiments PBMCs from MM and Pd patients were cultured in the presence of increasing concentration of anti-TRAIL monoclonal antibodies (mAb) (R&D Systems Inc.) (10 to 500 ng/ml) with or without RANKL (30 ng/ml).

3.3 ELISA assay

The levels of TRAIL were detected in the sera and in the media of PBMC cultures from the patients as well as from the same samples of control subjects using a human TRAIL enzyme-linked immunosorbent assay (ELISA) kit (supplied by Biomol Research Laboratories, Inc., Plymouth Meeting, PA, USA), according to the manufacturer's instructions. The levels of RANKL and OPG were detected in the media of PBMC cultures from the patients and controls by ELISA kits (Biomedica, GmbH, Wien, Austria). The samples were diluted to concentrations within the standard curve range. The absorption was determined with an ELISA reader at 450 nm (550 Microplate Reader Bio-Rad Laboratories Inc., CA, USA) and the results were expressed as mean \pm SE.

3.4 Statistical analyses

Statistical analyses were performed by Student *t* test with the Statistical Package for the Social Sciences (spssx/pc) software (SPSS, Chicago, IL). The results were considered statistically significant for *P* values less than .05.

4. RESULTS

4.1 TRAIL induces osteoclast differentiation in human PBMCs from healthy subjects

We investigated the effect of TRAIL on OC differentiation in PBMCs from healthy subjects. In control conditions monocyte precursors differentiate into OCs only in the presence of MCSF (25 ng/ml) and RANKL (30 ng/ml). In fact, within 18-21 days, multinucleated (more than 3 nuclei) and TRAP+ cells formed in cultures. When we added increasing doses of TRAIL to the PBMCs, cultured with MCSF in the absence of RANKL, we observed the formation of multinucleated TRAP+ cells in the presence of 100 and 200 ng/ml TRAIL, even if the obtained OCs were fewer with respect to the RANKL-treated cultures. On the contrary, when in PBMC cultures TRAIL and RANKL were simultaneously added with MCSF the OC differentiation did not occur (Figure 1). In addition, we observed differences in osteoclastic cell size obtained in the different culture conditions. In particular, TRAIL induced the formation of smaller multinucleated TRAP+ cells (Figure 2B), with respect to the larger OCs obtained in the presence of RANKL (Figure 2A), while no OCs were observed in the cultures simultaneously treated with RANKL and TRAIL (Figure 2C). In these last conditions the cells were viable, thus a toxic effect of the simultaneous addition of RANKL and TRAIL could be excluded. Moreover, OCs formed in the presence of TRAIL were functional as demonstrated by the ability to degrade mineralized matrix when plated onto commercial calcium phosphate film utilized as resorption substrate. In particular, resorbing areas formed by OCs induced by TRAIL were smaller (Figure 2E) with respect to the large resorption areas produced by OCs formed in the presence

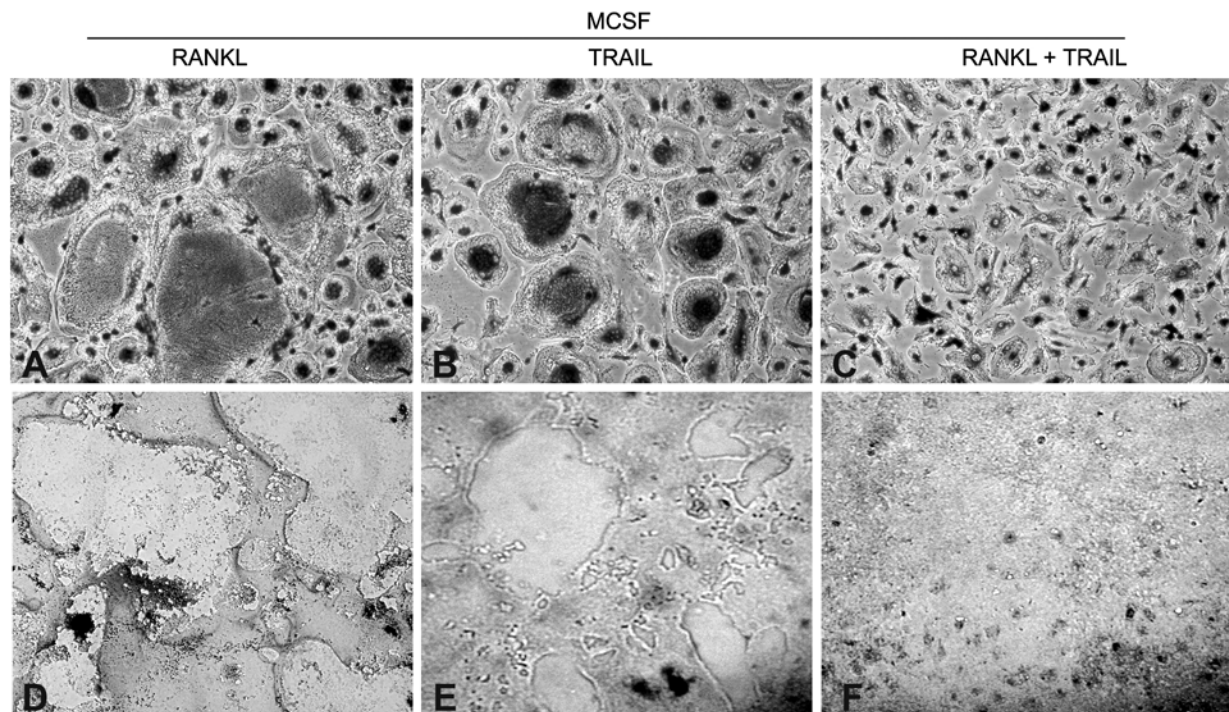


Figure 2. TRAIL induces the formation of functional osteoclasts from PBMCs of healthy subjects. (A) Representative field of TRAP⁺ multinucleated cells obtained from PBMCs cultured in the presence of M-CSF plus RANKL, (B) or plus 100 ng/ml TRAIL, (C) or plus TRAIL and 30 ng/ml RANKL. Photomicrographs of resorption areas formed on Millenium Osteologic slides by OCs from PBMCs cultured with M-CSF and RANKL (D) or TRAIL (E) or both TRAIL and RANKL (F).

of RANKL (Figure 2D). As expected no resorbing activity was observed in culture simultaneously treated with RANKL and TRAIL (Figure 2F).

4.2 TRAIL neutralization inhibits the formation of osteoclasts from PBMCs of MM and Pd patients

In our previous work, using an *in vitro* osteoclastogenesis model consisting of PBMCs from MM patients, we showed that OCs spontaneously arose in culture without the addition of exogenous cytokines, we also demonstrated the involvement of TRAIL in the elevated OC formation and high TRAIL levels in PBMC media from MM patients (22). Moreover, the spontaneous OC formation was also demonstrated in the same *in vitro* system from Pd patients and high TRAIL levels were detected in the sera from the same patients (28). To deeply understand the role of TRAIL in OC formation in pathological conditions with bone involvement such as MM and Pd patients, we performed osteoclastogenesis experiments by culturing PBMCs from MM and Pd patients in the presence of different concentrations of a neutralizing anti-TRAIL mAb. As shown in Figure 3A, our results confirmed the partial inhibition of OC formation in PBMCs from MM patients induced by the antibody, and interestingly we showed a similar effect in PBMCs from Pd patients. In fact, a statistically significant dose-dependent inhibition of spontaneous osteoclastogenesis in both culture systems was detected, even if the effect exerted by the antibodies was slightly lower in Pd with respect to the MM PBMCs, probably depending on the lower concentration of TRAIL in the media from PBMCs of Pd patients.

Interestingly, the anti-TRAIL dose-dependent inhibition of spontaneous osteoclastogenesis was completely abolished by the addition in the same culture system of 30 ng/ml RANKL (Figure 3B).

4.3 High TRAIL levels in the media of PBMC cultures and in the sera from patients with periodontal disease and multiple myeloma

In our previous works we found elevated levels of TRAIL in culture media from MM PBMC (22) and in the sera of Pd patients (28). To further compare the level of TRAIL in the two disease we detect TRAIL levels in the Pd PBMC culture media and in the sera of MM patients. In particular, we confirmed the elevated TRAIL levels in culture media from MM PBMC with respect to the control group 1 (42.86±0.57 pmol/L vs 20±1.9 pmol/L, p<0.001). Moreover, we detected higher TRAIL levels in culture media from PBMC of Pd patients than control group 2 (32.61±3 pmol/L vs 17±2 pmol/L, p<0.01), but they are lower than MM patients. Higher levels of RANKL and OPG were also found in MM and Pd PBMC media (data not shown).

Additionally, we confirmed the higher TRAIL serum levels in Pd patients with respect to control group 2 [812±199 pg/ml (range 410-1122) vs 329±261 pg/ml (range 30-769); p<0.001] and we found elevated serum levels in MM patients with respect to the control group 1 [862±386 pg/ml (range 550-2094 pg/ml) vs 812±199 pg/ml (range 410-1122); p< 0.0001] (Figure 4)

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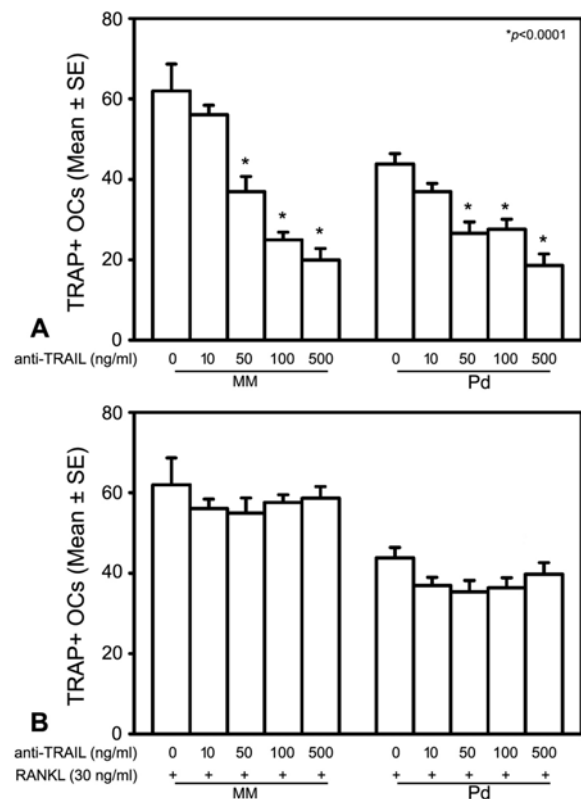


Figure 3. Neutralization of TRAIL inhibits osteoclastogenesis. PBMCs from MM and Pd patients were cultured in the presence of an anti-TRAIL mAb at different concentrations. The presence of anti-TRAIL mAb significantly reduced osteoclastogenesis in both culture systems (A). The addition of RANKL rescued the anti-TRAIL mAb inhibitory effect (B). The graphs represent the mean values \pm SE of a representative experiment.

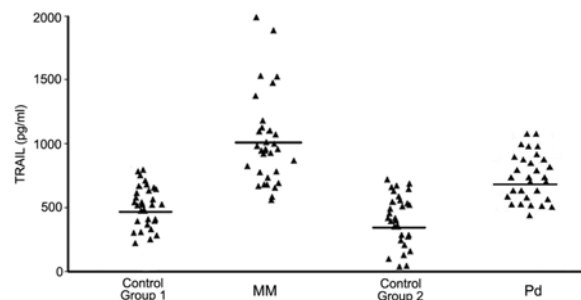


Figure 4. TRAIL levels are elevated in the sera of MM and Pd patients. TRAIL concentration was analyzed by ELISA assay in the sera collected from MM and Pd patients, as well as healthy donors distinguished as control group 1 and control group 2. MM and Pd patients had significantly higher serum levels of TRAIL compared to the controls.

5. DISCUSSION

Physiologically OCs, the cells responsible of bone resorption, originate from hematopoietic precursor cells under the tight control of RANK/RANKL/OPG

system (1). Abnormal osteoclastogenesis determines excessive bone resorption which can cause osteolytic bone diseases, including osteoporosis, arthritis, periodontal disease or hematological malignancies with associated bone lesions, such as MM. A series of cytokines, belonging to TNF superfamily molecules, mainly produced in pathological conditions, have been recently involved in modulating osteoclastogenesis. Many of these exert stimulatory effects on the formation of OCs, some others display inhibitory action on osteoclastogenesis, and others, such as TRAIL, interfere with OC differentiation in a not completely clear way, in fact conflicting data, including stimulatory or inhibitory action or lack of both have been published (12-15). The contradictory results reported on TRAIL and osteoclastogenesis can depend on diverse experimental approaches used by the different authors in their studies, different concentrations of the molecules, as well as various preparations of recombinant TRAIL used in the experiments. With the aim to contribute in the understanding of the interference of TRAIL with OC formation, in the present study we first investigate the effect of TRAIL on the differentiation of OCs in physiological condition and next in some pathological conditions with bone involvement utilizing osteoclastogenesis *in vitro* models, represented by PBMCs from healthy donors and patients affected by MM or Pd associated bone disease. We demonstrated that in PBMCs from healthy donors TRAIL can directly induce OC formation in the absence of RANKL while exert an inhibitory effect when added concomitantly to RANKL.

The stimulatory effect of TRAIL on osteoclastogenesis that we found is in agreement with the study published by Yen *et al.* in 2008, and suggests that this molecule is somehow a pro-osteoclastogenic factor in an *in vitro* system (13). On the other hand, the inhibitory effect we detected in the presence of RANKL is in line with data from Zauli *et al.* (12, 34) indicating that TRAIL inhibits the RANKL-induced OC formation. Thus taken together our findings and those from other authors, we propose that the opposite effects of TRAIL on the formation of OCs may be due to the possibility that the inhibitory action depends on the interference of TRAIL with other molecules, such as RANKL, in the absence of which TRAIL is able to exert a stimulatory effect through a not yet known mechanism.

However, the role of TRAIL on osteoclastogenesis could be much more complex than it appears from the data currently present in the literature, and it may be different in physiological or pathological conditions. This consideration comes from the data we show in the present paper, but begins from our previous work in which we demonstrated that in PBMCs from MM bone disease patients OCs spontaneously arose without the addition of exogenous cytokines possibly through the involvement of RANKL/OPG/TRAIL interaction (22), and other studies in which we also found the spontaneous OC formation from PBMCs of Pd patients which also had elevated serum levels of TRAIL (27, 28).

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Thus, in the present paper having first established that the levels of TRAIL, RANKL and OPG are elevated in the media of PBMCs from Pd patients, as occurred in those from MM patients that we previously demonstrated (22), we performed osteoclastogenesis experiments in the presence of anti-TRAIL mAb in the media of PBMCs from both patients. We found that the neutralization of TRAIL partially inhibits the spontaneous OC formation in a dose-dependent manner. This effect could be due to the interaction of TRAIL with OPG since we detect high levels of OPG in our culture media. In fact, when we neutralize TRAIL, OPG can become completely free to exert its natural effect as anti-osteoclastogenic factor by binding to RANKL. This becomes important considering that in our culture systems we detect large amount of RANKL, but in the presence of anti-TRAIL mAb RANKL fails to induce OC formation probably because bound by OPG. This finding is consistent with those of Vitovski *et al* demonstrating that TRAIL had the capacity to reverse the inhibitory action of OPG on RANKL-RANK binding by interacting with OPG (35). Thus, in our opinion OPG can represent the key molecule regulating TRAIL effect on osteoclastogenesis in pathological conditions. Moreover, the critical role of OPG/TRAIL interaction in determining the TRAIL effect on osteoclastogenesis is further confirmed by the demonstration that the addition in culture of RANKL completely rescue the inhibition of OC formation induced by the neutralization of TRAIL. This is also in agreement with data from Vitovski *et al* demonstrating that TRAIL, when present in excess over OPG, directly enhanced the binding of RANKL to its receptor RANK by titrating out the inhibitory molecule (35).

Moreover, high levels of TRAIL were also detected in the sera of Pd and MM patients with respect to the very low levels found in healthy donors. These findings highlight a critical role of TRAIL in the pathogenesis of osteolytic diseases, further supported by the high serum level of TRAIL in other pathologies with bone involvement, such as psoriatic arthritis, ankylosing spondylitis and rheumatoid arthritis (36-38).

Taken together all these findings we can conclude that the effect of TRAIL could be worthy of consideration in pathological conditions in which this factor is produced in large amount concomitantly to other molecules, rather than in physiological states in which ineffective levels of TRAIL can be detected. Moreover, TRAIL action is probably influenced by the complex network of cytokines that is established in the different diseases. However, further studies on the effect of TRAIL on OC formation in physiological conditions could help the understanding of molecular mechanisms regulating TRAIL role in pathological conditions.

6. ACKNOWLEDGMENT

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