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Osteoblasts Display Different Responsiveness to TRAIL-Induced Apoptosis During Their Differentiation Process

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Abstract Apoptosis can occur throughout the life span of osteoblasts (OBs), beginning from the early stages of differentiation and continuing throughout all stages of their working life. Here, we investigated the effects of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) on normal human OBs showing for the first time that the expression of TRAIL receptors is modulated during OB differentiation. In particular, the TRAIL receptor ratio was in favor of the deaths because of the low expression of DcR2 in undifferentiated OBs, differently it was shifted toward the decoys in differentiated ones. Undifferentiated OBs treated with TRAIL showed reduced cell viability, whereas differentiated OBs displayed TRAIL resistance. The OB sensitiveness to TRAIL was due to the up-regulation of DR5 and the down-regulation of DcR2. The main death receptor involved in TRAIL-reduced OB viability was DR5 as demonstrated by the rescue of cell viability observed in the presence of anti-DR5 neutralizing antibody. Besides the ratio of TRAIL receptors, the sensitivity of undifferentiated OBs to TRAIL-cytotoxic effect was also associated with low mRNA levels of intracellular anti-apoptotic proteins, such as cFLIP, the activation of caspase-8 and -3, as well as the DNA fragmentation. This study suggests that apoptotic effect exerted by TRAIL/TRAIL-receptor system on normal human OB is strictly dependent upon cell differentiation status.

Keywords Osteoblast · TRAIL · Apoptosis · TRAIL receptor · DcR2 · c-FLIP

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Introduction

Controlled cell death by apoptosis is recognized as an important component of embryogenesis and tissue morphogenesis, and, in adult skeletons it contributes substantially to physiological bone turnover, repair, and regeneration [1–3]. It is now generally accepted that the number of osteoblasts (OBs), the bone-forming cells, plays a critical role in bone turnover, and all major regulators of bone contribute to the regulation of OBs life span by modulating apoptosis [4].

In particular, apoptosis of OBs was reported during all physiological periods within their life span, beginning at the early stages of differentiation and continuing throughout the working life of this cell type [4]. It is believed that this process occurs in order to make space for advancing

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bone formation within the skeletal system. This was supported by the demonstration that once bone formation is completed, ~50–70 % of the OBs originally assembled at remodeling sites undergo apoptosis [4]. As apoptosis of bone cells is a rapid process and cannot be assessed in vivo during bone turnover in a normal adult organism [5], expression and function of apoptotic molecules, belonging to tumor necrosis factor (TNF) ligand and receptor families, have been investigated on bone cells in vitro, with controversial results from different experimental models and methods [1, 6, 7]. Among the members of the TNF family, some authors have examined the sensitivity of OBs to TNF-related apoptosis-inducing ligand (TRAIL), but contradictory results have also emerged from these studies [8–14].

TRAIL is a type II transmembrane protein that induces apoptosis through interactions with its two death domain-containing receptors, namely, DR4 and DR5 [15, 16]. Ligand-dependent activation of DR4 and DR5 involves receptor multimerization, with subsequent recruitment of Fas-associated protein with death domain that engages the initiator protease pro-caspase-8, via an interaction of their respective death effector domain [17]. TRAIL-activated caspase-8 subsequently leads to activation of downstream executioner caspases, including caspase-3 and -7, resulting in the cleavage of cellular substrates and ultimately cell death [18, 19]. However, DR4 and DR5 activity can be antagonized by the three so-called "decoy" receptor for TRAIL: DcR1 [20] and DcR2 [21], transmembrane receptors which lack functional death domains, and osteoprotegerin [22], a soluble molecule capable of binding to TRAIL. Consequently, a perturbation in the ratio of TRAIL decoy and death receptors is implicated in determining the responsiveness of cells to TRAIL. Furthermore, susceptibility to TRAIL-induced apoptosis can also be regulated by the levels of intracellular anti-apoptotic molecules, such as cellular FLICE-like inhibitory protein (cFLIP) and X-linked inhibitor of apoptosis proteins (XIAP). The role of c-FLIP in abrogating TRAIL-mediated apoptosis is due to its structural similarity to caspase-8. Indeed, by blocking the autoproteolytic cleavage of pro-caspase-8 cFLIP halts the transduction of death signal, and high levels of cFLIP lead to lack of sensitivity to TRAIL in several cancer types [23–27]. Moreover, XIAP which is the most potent inhibitor of apoptosis proteins among the IAPs can bind and inactivate caspase-3, -7, and -9 [28], and high XIAP expression has been postulated as a mechanism of resistance to TRAIL apoptotic effect in different cancer cells [29–32]. (shortened).

At present, very few evidences have been published about the role of TRAIL on normal OBs demonstrating that these cells express all TRAIL receptors and are resistant to its apoptotic effect [13]. Moreover, TRAIL –/– deficient mice do not show overt differences in gross density of bones [33]. Therefore, on the bases of literature findings it emerges that the effect of TRAIL on osteoblastic cells, either normal or transformed, could be more complex than it appears and becomes critically relevant to take into account the profound functional changes to which OBs undergo during their differentiation. Thus, in this study we investigated whether the sensitivity of OBs to TRAIL-mediated apoptosis could vary during their differentiation process and we also analyzed the mechanism/s responsible of TRAIL effect in undifferentiated and differentiated normal human OBs.

Materials and Methods

Ethics Statement

This study was approved by the Ethical Committee of Tor Vergata University Hospital (Protocol number 121/06 of 20 December 2006). All study participants provided written informed consent.

Human OBs

Trabecular bone specimens, obtained from healthy subjects, who undergo to femur surgery following traumatological events were provided by the Orthopaedic Department of Tor Vergata. These specimens were cleaned off soft tissues, reduced to small fragments, and digested with 0.5 mg/mL *Clostridium histolyticum* neutral collagenase (Sigma Chemical Co., St. Louis, MO, USA) in phosphate-buffered saline (PBS) with gentle agitation for 30 min at 37 °C. Bone fragments were then washed (three times) with minimum essential medium (α-MEM) (Gibco Ltd., Uxbridge, UK) containing 3.024 g/L sodium bicarbonate, and cultured in medium supplemented with 10 % fetal calf serum (Gibco Ltd.), 100 IU/mL penicillin, 100 mg/mL streptomycin, 2.5 mg/mL amphotericin B, and 50 IU/mL mycostatin, at 37 °C in a water-saturated atmosphere containing 5 % CO_2. Cells were fed by medium replacement every 3–4 days. In these conditions, the osteoblast resident in the explant proliferated and migrated to the culture substrate, reaching confluence within 3–4 weeks. Cells were then trypsinized and transferred into appropriate culture dishes for characterization and experiments.

Cell Viability Assay

Mitochondrial dehydrogenases activity was determined by MTT-assay. This assay is based on the ability of formazan dye crystals to develop only in living cells, providing an indication of the mitochondrial integrity and activity

which, in turn, may be interpreted as a measure of cell viability. Osteoblasts were cultured in 96-well tissue-culture plates; after having reached the confluence, part of the cells were stopped and used as Time 0 (t_0) while the others were grown with the same medium supplemented with 50 μg/mL ascorbic acid and Dexametasone 10^{-8} M for 3, 7, 10, and 20 days (t_3, t_7, t_{10}, and t_{20}) to obtain differentiation. Cells were treated with different concentrations of rh-TRAIL (10–500 ng/mL, TRAIL/TNFSF10, R&D Systems Inc., MN, USA) for 24 and 48 h to evaluate the cell viability, on days 0, 10, and 20. The cell viability experiments were performed in the presence of 10 % FBS. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) 0.5 mg/mL were added to the culture media followed by 4 h incubation at 37 °C in a humidified 5 % CO_2 atmosphere. The reaction was stopped by adding 150 μL of 0.04 N HCl in absolute isopropanol. The optical density was read at 570 nm using an automatic plate reader (550 Microplate Reader Bio-Rad Laboratories Inc., CA, USA). The results were compared to cells incubated under control conditions. In parallel, the effect of 100 ng/mL TRAIL on OBs viability was evaluated in cells pretreated for 30 min with 5 μg/mL of antagonist anti-DR5 or anti-DR4 neutralizing monoclonal antibodies (mAbs) (Alexis, San Diego, CA, USA) or an irrelevant anti-IgG.

DNA Fragmentation

Human OBs were cultured in 6-well tissue-culture plates until reaching the confluence in α-MEM supplemented with 10 %. DNA extracts were prepared from OBs treated with rh-TRAIL at a concentration of 100 ng/mL for different times. DNA was purified using the Apoptotic DNA Ladder Kit (Roche Applied Science, Mannheim, Germany), to detect the typical apoptotic DNA fragments (ladder), according to the manufacturer's instructions. The kit provides a lyophilized apoptotic U937 cell sample as positive control. Samples were then electrophoresed on 2 % agarose gel containing 0.01 % ethidium bromide, and the resulting bands were detected by a light sensitive CCD video system (BioDocAnalyze, Whatman Biometra, Göttingen, Germany).

RNA Isolation and Real-Time PCR Analysis

OBs were seeded in 6-well tissue-culture plates; after having reached the confluence, part of the cells were stopped and used as Time 0 while the others were grown with the same medium supplemented with 50 μg/mL ascorbic acid and Dexametasone 10^{-8} M for 3, 7, 10, and 20 days to obtain differentiation. At the indicated times, OB cultures were subjected to mRNA extraction using spin columns (RNeasy, Qiagen, Hilden, Germany) to detect the expression of cFLIP, XIAP, and the housekeeping genes glyceraldehyde phosphate dehydrogenase (GAPDH). First-strand cDNA was generated using the SuperScript First-Strand Synthesis System kit for RT-PCR (Invitrogen, Carlsbad, CA, USA). Briefly for the first-strand cDNA synthesis, an RT mixture containing 1 μg total RNA, dNTPs, Oligo(dT), RT buffer, $MgCl_2$, DTT, RNaseOUT, SuperScript II RT, DEPC-treated water to final volume 100 μL was prepared, according to the manufacturer's instructions. cDNA was amplified with the iTaq SYBR Green supermix with ROX kit (Bio-Rad Laboratories, Bio-Rad Laboratories Inc., CA, USA), and the PCR amplification was performed using the Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories). The following primer pairs were used for the PCR amplification: cFLIP (S: 5′-AAGCTGTCTGTCGGGGACTT-3′; AS: 5′-GGTG GGTCTCCACAGCTTTT-3′); XIAP (S: 5′-GACAGTATG CAAGATGAGTCAAGTCA-3′; AS: 5′-GCAAAGCTTCC TCCTCTTGCAG-3′); osteocalcin (S: 5′-ACACTCCTCG CCCTATTG-3′; AS: 5′-CAGCCATTGATACAGGTAG C-3′); alkaline phosphatase (Ph Alk) (S: 5′-CGCACGG AACTCCTGACC-3′; AS: 5′-GCCACCACCACCATC TCG-3′); osteopontin (S: 5′-CTGATGAATCTGATGAA CTGGTC-3′; AS: 5′-GTGATGTCCTCGTCTGTAGC-3′); GAPDH (S: TCATCCCTGCCTCTACTG; AS: TGCTTC ACCACCTTCTTG). The running conditions were incubation at 95 °C for 3 min, and 40 cycles of incubation at 95 °C for 15 s and 60 °C for 30 s. After the last cycle, the melting curve analysis was performed into 55–95 °C interval by incrementing the temperature of 0.5 °C. The fold change values were calculated by Pfaffl method [34].

Western Blot Analysis

To study the expression of TRAIL-Rs during OB differentiation, cells were seeded in 6-well tissue-culture plates; after having reached the confluence, part of the cells were lysed and used as Time 0 while the others were grown with the same medium supplemented with 50 μg/mL ascorbic acid and Dexametasone 10^{-8} M for 3, 7, 10, and 20 days to obtain differentiation. At the indicated times, OBs were lysed by incubation on ice for 30 min in lysis buffer containing 50 mmol/L Tris–HCl (pH 8.0), 150 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid, 1 % NP40, and 1 mmol/L phenylmethyl sulfonyl fluoride.

To study caspase-8 and -3 activation, OBs were seeded in 6-well tissue-culture plates; after having reached the confluence, the cells were treated with 100 ng/mL rh-TRAIL for 0–8 h. Cells were lysed by incubation on ice for 30 min in lysis buffer containing 20 mM Tris–HCl (pH 7.5), 1 % Triton X-100, 150 mM NaCl, 10 % glycerol, 1 mM Na_3VO_4, 50 mM NaF, 100 mM phenylmethylsulfonyl fluoride, and a commercial protease inhibitor mixture

(Sigma Aldrich). About 30 μg of cell proteins was subjected to 12 % SDS-PAGE gel and subsequently transferred to nitrocellulose membranes (Hybond, Amersham Pharmacia, London, UK). The blots were probed overnight at 4 °C with mouse anti-caspase 3 (Cell Signaling, San Diego, CA, USA) and anti-β-actin monoclonal Abs (Chemicon International Inc.), rabbit anti-DR4, anti-DR5, anti-DcR1, anti-DcR2 (Abcam, Cambridge, UK), anti-caspase 8 (BD Pharmingen, San Diego, CA, USA), anti-cFLIP and anti-XIAP (Cell Signaling Technology, Danvers, MA, USA) polyclonal Abs. After incubation with the appropriate fluorescent-dye-conjugated secondary Ab (LI-COR Biosciences GmbH Bad Homburg, Germany), specific reactions were revealed with the LI-COR's Odyssey Infrared Imaging System (LI-COR Biosciences-Biotechnology, Lincoln, NE, USA). Densitometric analysis was performed using LI-COR's Odyssey Infrared Imaging System Application Software version 3.0 (LI-COR Biosciences-Biotechnology).

Statistical Analyses

Statistical analyses were performed by Student's t test with the Statistical Package for the Social Sciences (spssx/pc) software (SPSS, Chicago, IL, USA). A value of $p < 0.05$ was taken to be statistically significant.

Results

Sensitivity of OBs to TRAIL-Mediated Apoptosis

Sensitivity to TRAIL in human OBs was investigated by analyzing cell viability through MTT-assay in undifferentiated and differentiated OBs in the presence of TRAIL. OBs were cultured in 96-well tissue-culture plates; after the cells reached the confluence, part of them have been used as undifferentiated cells (t_0 OBs) while the others were grown with osteogenic medium for 3 (t_3 OBs), 7 (t_7 OBs), 10 (t_{10} OBs), and 20 (t_{20} OBs) days to induce their osteoblastic differentiation. All the cells were first characterized for their osteoblastic parameters (alkaline phosphatase, osteocalcin, and osteopontin), which were weakly expressed at t_0, slightly increased at t_3, and significantly enhanced at t_7, t_{10}, and t_{20} (Supplementary Fig. 1 shows the data of t_0, t_{10}, and t_{20}). All the OBs were treated with increasing concentrations of rh-TRAIL (ranging from 10 to 500 ng/mL) for 24 and 48 h, and the viability of OBs was determined in both TRAIL-treated and untreated cells as control. As shown in Fig. 1a, the viability of t_0 OBs was reduced by TRAIL in a dose- and time-dependent manner. In particular, when OBs were exposed to 10 ng/mL rh-TRAIL for 24 h their viability was slightly but significantly decreased compared to untreated OBs. TRAIL treatment further reduced the viability of t_0 OBs at 25 and 50 ng/mL, the maximum decrease was reached at 100 ng/mL TRAIL, and no additional decline was observed in the presence of higher concentrations of the molecule. After 48 h TRAIL still induced a dose- and time-dependent decrease of t_0 OB viability, but the effect was more pronounced with respect to what was observed at 24 h. In fact, the reduction of cell viability in the presence of 100 ng/mL TRAIL for 24 h was of about 30 %, while the same concentration after 48 h induced a decrease of about 48 % with respect to the control (Fig. 1a). Surprisingly, we found that TRAIL fails to induce any effect on cell viability of differentiated OBs, either for early (t_3 and t_7 OBs, data not shown) or late differentiated cells (t_{10} and t_{20} OBs) after both 24 and 48 h TRAIL treatment with respect to untreated cells (Fig. 1b, c).

Expression of TRAIL-Receptors During Osteoblast Differentiation

On the basis of the described results, we investigated whether the OB differentiation process could induce a different expression of death and decoy TRAIL receptors. We found that undifferentiated and differentiated OBs express all TRAIL receptors at protein levels. In particular, the expression of the decoy receptor DcR2 was low in t_0 OBs, and increased in a time-dependent manner during the differentiation process, while the levels of DcR1 were not modified (Fig. 2). In addition, the expression of both death TRAIL receptors, DR4 and DR5, did not display any significant variation during the OB differentiation (Fig. 2). These findings indicated that only the variation of DcR2 expression took place during OB differentiation. Consequently, the ratio between death and decoy TRAIL receptor was in favor of the death receptors in undifferentiated OBs, thereby contributing to their sensitivity to TRAIL effect in reducing their cell viability. Differently, the ratio was shifted in favor of decoy receptors during and after OB differentiation, explaining the lack of TRAIL effect on the viability of these cells as shown in Fig. 1.

DR5 Involvement in Osteoblast Apoptosis

In order to identify which receptor could be involved in TRAIL-mediated effect on undifferentiated OBs, we studied the expression of death and decoy TRAIL receptors after cell treatment with 100 ng/mL TRAIL at different times (0–0.5–1–2–4–6–8 h). In Fig. 3, we showed that in undifferentiated OBs TRAIL treatment determined the reduction of DcR2 and the increase of DR5 expression. In particular, DcR2 levels significantly declined after 2 h TRAIL treatment, this reduction became more pronounced after 4 h, and remained reduced compared to control up to

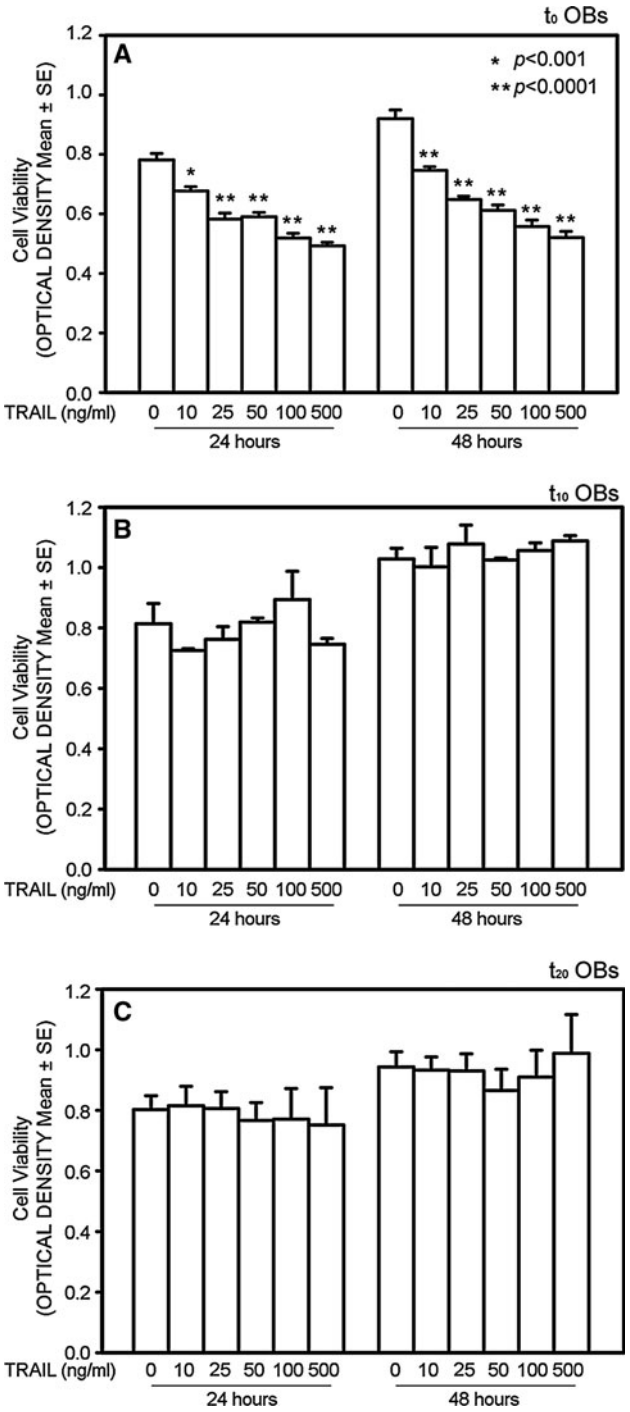

Fig. 1 Sensitivity of OBs to TRAIL-mediated apoptosis. Undifferentiated (t_0 OBs) and differentiated OBs (t_{10} and t_{20} OBs), treated with increasing concentrations of rh-TRAIL (ranging from 10 to 500 ng/mL) for 24 and 48 h, were cultured in 96-well tissue-culture plates. **a** t_0 OB viability was reduced in a dose- and time-dependent manner by TRAIL treatment after both 24 and 48 h. **b**, **c** The viability of both t_{10} and t_{20} OBs was not affected by the presence of TRAIL. The graphs represent the mean values ± SE of optical density at 570 nm of five independent experiments in which each treatment was performed in quadruplicate

8 h of treatment. On the contrary, the levels of DR5 increased after 1 h of treatment, reached the maximum levels after 2 h exposure to TRAIL, and remained higher up to 8 h treatment respect to DR5 baseline expression. In the same experiments, we demonstrated that TRAIL treatment did not modify the expression pattern of DcR1 and DR4 (data not shown). On the basis of these findings, we investigated OB viability in the simultaneous presence of TRAIL and anti-DR5 or anti-DR4 neutralizing antibodies or both. We demonstrated that the reduction of t_0 OB viability induced by TRAIL after 48 h was partially abolished only in the presence of anti-DR5 neutralizing antibody. No effect on the viability of TRAIL-treated OBs was exerted by anti-DR4 neutralizing antibody. Anti-DR4 or -DR5 as well as anti-IgG antibody was utilized as negative controls (Fig. 4).

Expression of cFLIP and XIAP on OBs During Their Differentiation

The described different sensitivity of undifferentiated and differentiated OBs to TRAIL lets us to study the different expression of the intracellular anti-apoptotic molecules cFLIP and XIAP during OB differentiation. By means of real-time PCR and western blot we showed that the expression levels of cFLIP increased during OB differentiation. In particular, the lowest mRNA levels of cFLIP were found in t_0 OBs; in comparison with these, cFLIP mRNA levels were unchanged in t_3 OBs, displayed 1.8- and 2.8-fold change increase in t_7 OBs ($p < 0.001$) and t_{10} OBs ($p < 0.0001$), respectively, and no further enhancement was detected until the 20th day of cell differentiation (t_{20} OBs) (Fig. 5a). Instead, the gene expression of XIAP did not undergo significant changes during differentiation (Fig. 5b). The detection of protein levels of both cFLIP and XIAP overlaps the results of gene expression, in fact cFLIP increased during the differentiation process, while XIAP levels were not modified (Fig. 5c, d). Our results therefore demonstrate that differentiated OBs were protected from TRAIL-mediated apoptosis by the increase of cFLIP, which is an intracellular molecule inhibiting caspase-8.

TRAIL Activates Caspase-8 and -3 in Undifferentiated OBs

On the basis of the low expression of cFLIP in undifferentiated OBs, we investigated the activation of caspase-8 and -3.

As is well known, caspase-8 is the initial caspase activated during TRAIL-induced apoptosis in other cell types [17–19]. Its activation consists of as first cleavage of

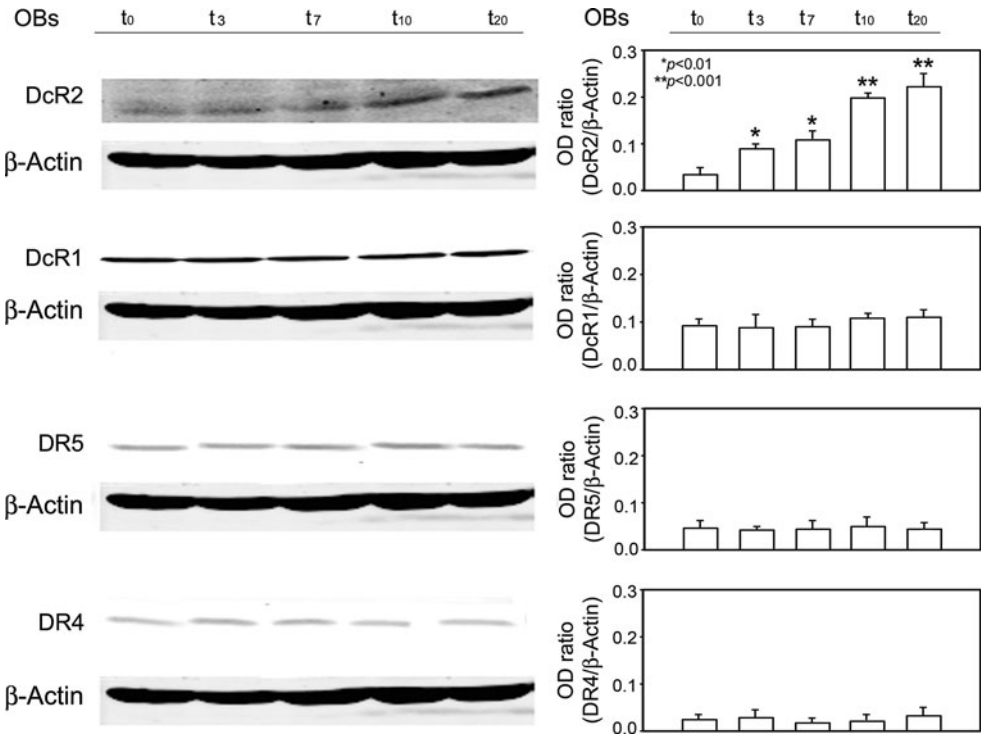

Fig. 2 TRAIL-receptor expression during osteoblast differentiation. Western blot analysis has been performed on undifferentiated (t_0 OBs) and differentiated OBs (t_3, t_7, t_{10}, and t_{20} OBs) to detect the expression of death and decoy TRAIL receptors. The histograms report the intensity of the bands quantified by densitometry and normalized to β-Actin. One of four independent experiments is also shown

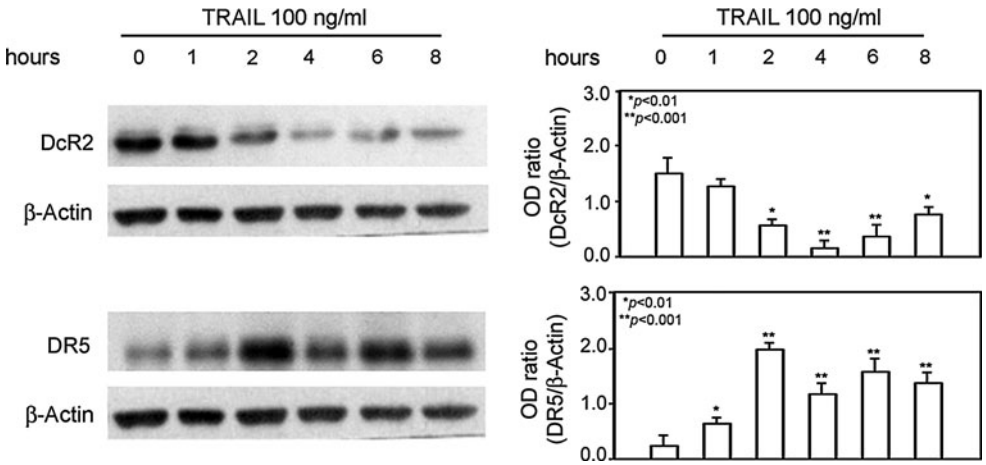

Fig. 3 TRAIL regulates DcR2 and DR5 expression in human undifferentiated OBs. Undifferentiated OBs, treated with 100 ng/mL TRAIL at the indicated times, were lysed and analyzed by western blot analysis to detect the protein levels of TRAIL receptors. The expression of DcR2 and DR5 was modulated by TRAIL treatment. The intensity of the bands obtained by western blot analysis was quantified by densitometry (histogram) and normalized to β-Actin. One of five independent experiments is also shown

procaspase-8 which generates two small fragments, p40 and p36 intermediate forms, and a second cleavage resulting in the formation of the p23 active fragment. By western blot analysis, we demonstrated the activation of caspase-8 in undifferentiated OBs treated with 100 ng/mL TRAIL from 0.5 up to 8 h treatment. In particular, the generation of intermediate forms (p40, p36) could be observed already after 2 h TRAIL treatment, and after 4 h a second cleavage took place, inducing formation of the p23 active caspase fragment (Fig. 6a). It is also known that activated caspase-8 cleaves caspase-3. Thus, we examined whether the cleavage of caspase-3 could be induced by TRAIL treatment. As shown in Fig. 6b, the p17 cleaved form of caspase-3 was detected in undifferentiated OBs after 4, 6, and 8 h TRAIL treatment. The activation of caspases is followed by DNA fragmentation. In particular, following the addition of TRAIL to the culture media, the formation of the DNA ladder was clearly evident in OBs after 8 and 15 h incubation (data not shown).

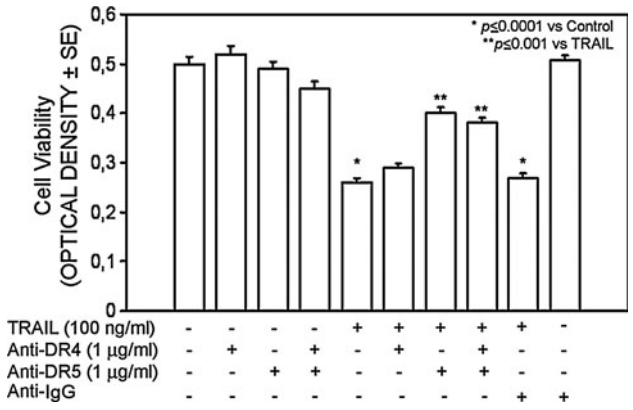

Fig. 4 Effect of neutralizing death receptor antibodies on osteoblast viability treated with TRAIL. Undifferentiated OBs were cultured in control conditions (−), in the presence of 100 ng/mL TRAIL (+), with (+) or without (−) anti-DR4, anti-DR5 neutralizing antibodies or anti-IgG control Ab, and the cell viability was assessed by MTT-assay. OBs were pre-treated for 30 min with the indicated antibodies before the addition of TRAIL. The graph represents the mean values ± SE of optical density at 570 nm of five independent experiments in which each treatment was performed in quadruplicate

Discussion

In this study, we investigated the effects of TRAIL on normal human OBs showing for the first time that their responsiveness to TRAIL varies during the differentiation process. We demonstrated that undifferentiated OBs are sensitive, whereas differentiated ones are resistant to TRAIL-apoptotic effect. Up to now, literature data have reported conflicting results concerning the apoptotic role of TRAIL in osteoblastic cells [8–14]. In particular, it has been demonstrated that some osteosarcoma cell lines are resistant to TRAIL apoptotic effect while others are sensitive [8, 11]. In addition, their responsiveness to TRAIL could also change, this is the case of the BTK-143 osteogenic sarcoma cells, which can gradually acquire TRAIL resistance due to progressive acquisition of the DcR2 [12], or that of MG 63 osteosarcoma cells which undergo apoptosis in response to DR5 agonist [10]. Furthermore, other investigators have reported the resistance of OB-like cells from osteoarthritic patients or normal human OBs to TRAIL-mediated apoptotic effect [8, 13]. Differently, normal human OBs infected with *Staphylococcus aureus* or co-cultured with myeloma cells become sensitive to TRAIL-mediated apoptosis [13, 14].

The different behavior observed by the authors on osteoblastic cells in response to TRAIL could be related to the different models used in the mentioned studies, and particularly the data obtained on normal human OBs, displaying resistance to TRAIL-induced apoptosis, did not consider the stage of OB differentiation. This is of particular importance since OBs undergo profound morphological and

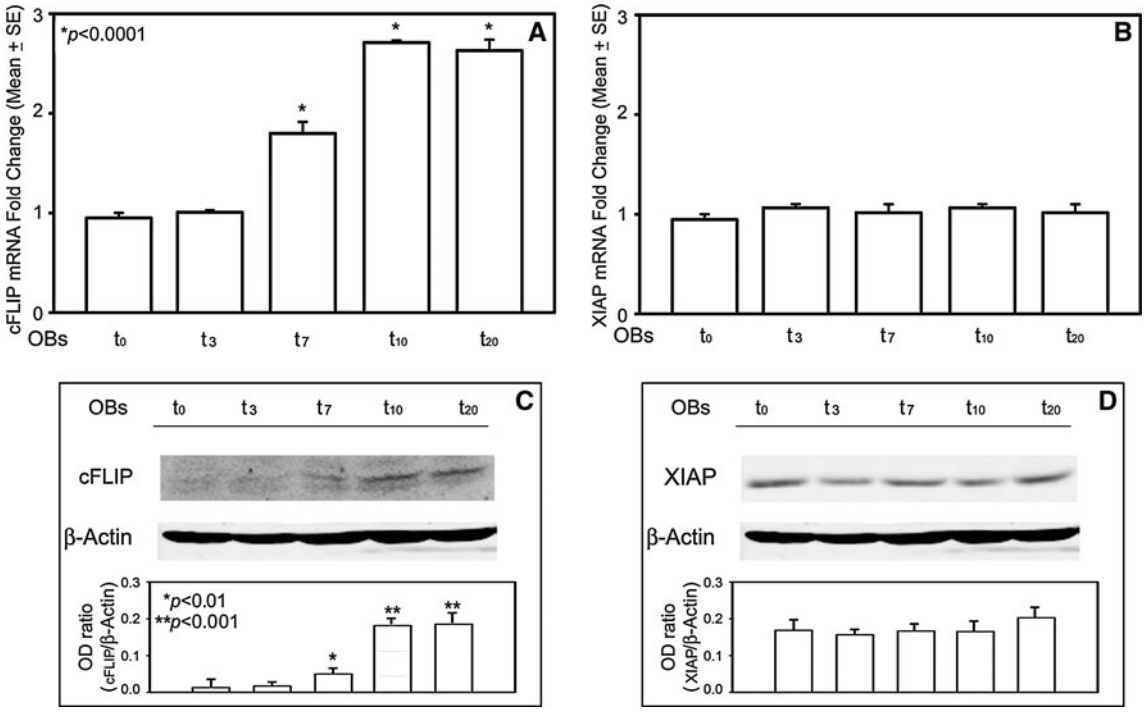

Fig. 5 Expression of cFLIP and XIAP on OBs during their differentiation. Undifferentiated (t_0 OBs) and differentiated OBs (t_3, t_7, t_{10}, and t_{20} OBs), OBs were cultured in 6-well tissue-culture plates, and RNA extract and protein lysates were subjected to real-time PCR and western blot analysis. Differential expression of cFLIP was demonstrated during the osteoblast differentiation at mRNA (**a**) and protein level (**c**), while no changes were found in XIAP levels (**b**, **d**). One of four independent experiments is also shown

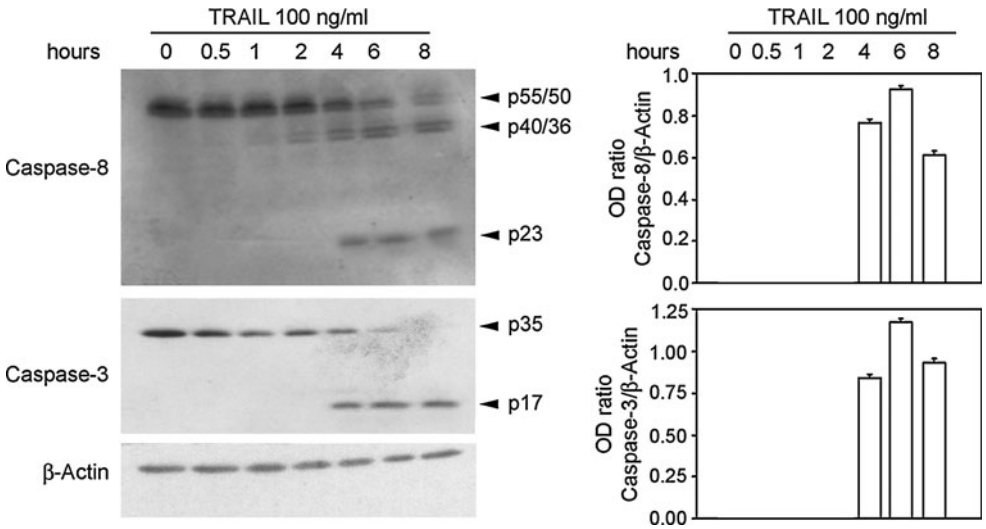

Fig. 6 TRAIL activates caspase-8 and -3 in undifferentiated OBs. Undifferentiated OBs, treated with 100 ng/mL TRAIL, were lysed at the indicated times and analyzed by western blot analysis to detect the protein levels of inactive and active forms of caspase-8 and -3. The fragments at 23 kDa and 17 kDa represent the active cleaved form for caspase-8 and -3. The intensity of the bands obtained by western blot analysis was quantified by densitometry (histogram) and normalized to β-Actin. One of five independent experiments is also shown

functional changes during their differentiation process, starting from the commitment of osteoprogenitor cells, their differentiation into pre-OBs and finally in mature OBs [35]. Thus, it becomes very important to take into account in which stage of cell differentiation TRAIL effects are studied in normal OBs. For this reason, we investigated whether the induction of OB differentiation can determine by itself a different responsiveness of OBs to TRAIL treatment. Interestingly, we found that only undifferentiated normal human OBs are sensitive to TRAIL-induced apoptosis in a time- and dose-dependent manner; on the contrary, differentiated OBs become resistant as demonstrated by the lack of TRAIL effect on their viability. Furthermore, we showed that the different behavior of undifferentiated and differentiated OBs to TRAIL treatment is associated to the variation of the ratio between death and decoy TRAIL receptors, which is critical in determining the fate of the cells. Indeed, we found that the induction of OB differentiation induced the increase of the DcR2 expression respect to undifferentiated OBs, whereas DR5, DR4, and DcR1 expressions were not modified during the differentiation process. These findings indicated that in undifferentiated OBs the ratio of TRAIL receptors was shifted toward the deaths, making these cells sensitive to TRAIL-apoptotic effect. On the contrary, in differentiated OBs the receptor ratio was moved in favor of the decoys, thereby protecting these cells from TRAIL-mediated apoptosis. In addition, we demonstrated that undifferentiated OBs respond to TRAIL by further pushing the receptor ratio in favor of those of deaths with the up-regulation of DR5 and the down-regulation of DcR2, suggesting that DR5 can be the main receptor by which TRAIL triggers the apoptotic effect. This was also sustained by the demonstration that only anti-DR5 neutralizing antibody almost completely restores the TRAIL-induced reduction of OB viability. Our findings indicate that in physiological conditions OBs acquiring a different expression of DcR2 and DR5 receptors vary their sensitivity to TRAIL. This is particularly relevant if we consider literature data, showing that in pathologic osteoblastic cells lines the same receptors modulate cell responsiveness to TRAIL [10, 12].

However, cell-resistance or -sensitivity to TRAIL-mediated apoptosis is at least only in part determined by the balance of its death and decoy receptors, as it can also be correlated to the levels of some intracellular anti-apoptotic molecules, such as cFLIP. Thus, we subsequently evaluated the expression of cFLIP during OB differentiation. Interestingly, we showed low cFLIP mRNA and protein levels in undifferentiated OBs and its higher expression in differentiated OBs, indicating that, besides the different receptor ratio, the sensitivity or resistance to TRAIL-apoptotic effect is also associated to the varied c-FLIP expression during OB differentiation process. Our findings are in line with those from other authors demonstrating that the expression of cFLIP was highest in the TRAIL-resistant melanomas, while low or undetectable in the TRAIL-sensitive melanomas [26], and data from other investigators showing that high levels of cFLIP lead to lack of sensitivity to TRAIL in several cancer types [23–27]. Moreover, according with the low levels of cFLIP in undifferentiated OBs, we demonstrated in these cells the activation of caspase-8, the cleavage of caspase-3, and DNA fragmentation, which represent the intracellular events of the typical apoptotic pathway activated by TRAIL in other cells.

In conclusion, our results highlight a different responsiveness of human OBs to TRAIL: sensitivity of undifferentiated OBs to TRAIL cytotoxic effect and resistance of fully differentiated ones. These findings could be of relevant importance in determining the correct number of working OBs which are needed to build new bone. Moreover, in pathological conditions, in which the overexpression of TRAIL has been demonstrated [36–41], our data suggest that TRAIL could contribute to the impairment of OB differentiation by targeting the apoptosis of OB precursors.

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