International Journal of Cardiology 169 (2013) 296–304

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Aortic valvular interstitial cells apoptosis and calcification are mediated by TNF-related apoptosis-inducing ligand

Antonella Galeone [a,b,1], Giacomina Brunetti [b,*], Angela Oranger [b], Giovanni Greco [b], Adriana Di Benedetto [b], Giorgio Mori [c], Silvia Colucci [b], Alberta Zallone [b], Domenico Paparella [a], Maria Grano [b]

[a] Department of Emergencies and Organ Transplantation (DETO), Division of Cardiac Surgery, University "Aldo Moro" of Bari, Italy
[b] Department of Basic Medical Sciences, Neurosciences and Sense Organs, Section of Human Anatomy and Histology, University "Aldo Moro" of Bari, Italy
[c] Department of Clinical and Experimental Medicine, University of Foggia, Italy

ARTICLE INFO

Article history:
Received 11 April 2013
Received in revised form 30 August 2013
Accepted 27 September 2013
Available online 5 October 2013

Keywords:
Aortic calcification
Apoptosis
TRAIL

ABSTRACT

Background/Objectives: Calcific aortic valvular disease (CAVD) is an actively regulated process characterized by the activation of specific osteogenic signaling pathways and apoptosis. We evaluated the involvement in CAVD of the TNF-related apoptosis-inducing ligand (TRAIL), an apoptotic molecule which induces apoptosis by interacting with the death receptor (DR)-4 and DR5, and whose activity is modulated by the decoy receptor (DcR)-1 and DcR2.
Methods: Sections of calcific and normal aortic valves, obtained at surgery time, were subjected to immunohistochemistry and confocal microscopy for TRAIL immunostaining. Valvular interstitial cells (VICs) isolated from calcific (C-VICs) and normal (N-VICs) aortic valves were investigated for the gene and protein expression of TRAIL receptors. Cell viability was assayed by MTT. Von Kossa staining was performed to verify C-VIC ability to produce mineralized nodules. TRAIL serum levels were detected by ELISA.
Results: Higher levels of TRAIL were detected in calcific aortic valves and in sera from the same patients respect to controls. C-VICs express significantly higher mRNA and protein levels of DR4, DR5, DcR1, DcR2 and Runx2 compared to N-VICs. C-VICs and N-VICs, cultured in osteogenic medium, express significantly higher mRNA levels of DR4, Runx2 and Osteocalcin compared to baseline. C-VICs and N-VICs were sensitive to TRAIL-apoptotic effect at baseline and after osteogenic differentiation, as demonstrated by MTT assay and caspase-3 activation. TRAIL enhanced mineralized matrix nodule synthesis by C-VICs cultured in osteogenic medium.
Conclusions: TRAIL is characteristically present within calcific aortic valves, and mediates the calcification of aortic valve interstitial cells in culture through mechanism involving apoptosis.

1. Background/Objectives

Aortic stenosis (AS) is the most common among heart valve diseases (43.1%) [1] and its prevalence is around 2% and increases with age [2–4]. Degenerative aetiology is predominant (81.9%) [1], however calcific aortic valve disease (CAVD) can no longer be considered a passive process in which the valve degenerates with age in association with calcium accumulation. Instead, CAVD appears to be an actively regulated process including chronic inflammation, lipoprotein deposition, renin–angiotensin system involvement, extracellular matrix remodeling, activation of specific osteogenic signaling pathways involved in the differentiation of the valvular interstitial cells (VICs) from fibroblast/myofibroblast to an osteoblast-like phenotype [5] and apoptosis [6]. Briefly an endothelial injury or disruption may allow circulating lipids, including low-density lipoprotein (LDL) and lipoprotein (a), to enter the valvular interstitial tissue [7] where they undergo oxidative modification [8]. These oxidised lipoproteins are highly cytotoxic and capable of stimulating inflammatory activity and mineralisation; both macrophages and activated T lymphocytes are present in the early and advanced lesions of aortic valves [9,10] and release enzymes, such as matrix metalloproteinases, that cause degradation of collagen, elastin and proteoglycans within the aortic valve cusps [11]. Mineralization arises in close proximity to areas of inflammation and has been demonstrated in early [10] as well as advanced lesions [12]. Several features suggest the presence of an active highly regulated process closely resembling developmental bone formation [13,14]. *In vitro* studies of cultured explants of stenotic valves have identified cells with osteoblastic characteristics capable of phenotypic differentiation and spontaneous calcification [15]. These osteogenic cells or 'calcifying valvular

* Corresponding author at: Department of Basic Medical Sciences, Neurosciences and Sense Organs, Section of Human Anatomy and Histology, University "Aldo Moro" of Bari, Piazza Giulio Cesare 11, 70124 Bari, Italy. Tel.: +39 0805478306; fax: +39 0805478361.
E-mail address: giacomina.brunetti@uniba.it (G. Brunetti).
[1] PhD School in "Cellular sciences and technologies", Department of Basic Medical Sciences, Section of Human Anatomy and Histology, University "Aldo Moro" of Bari, Italy.

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http://dx.doi.org/10.1016/j.ijcard.2013.09.012

cells' express and produce a variety of regulatory bone matrix proteins including osteopontin (OPN) [16,17] and bone morphogenetic proteins (BMPs) [12]. The initiation of mineralization (nucleation) may be stimulated by the presence of oxidized lipids [8,12] or by the presence of cellular degradation products following apoptosis [18]. To our knowledge the transforming growth factor (TGF)-β1 is the only pro-apoptotic cytokine that has been detected in human calcific aortic valves [6] while the presence of TNF-related apoptosis-inducing ligand (TRAIL) has not yet been assessed. TRAIL belongs to the TNF super-family and induces apoptosis by interacting with its death domain-containing receptors, DR4 [19] and DR5 [20], but its activity can be modulated by two decoy receptors DcR1 [20] and DcR2 [21] which lack death domain and cannot induce apoptosis. TRAIL induces cell death in a wide variety of tumor cell lines [22] however increasing experimental evidence shows that TRAIL exhibits regulatory role in various normal tissues as well [23]. In the present study, we evaluated a series of human calcified and non calcified aortic valves for the immunohistochemical presence of TRAIL, and assessed the influence of TRAIL and its receptors on valvular calcification and the development of an osteoblast-like phenotype in cultured human aortic VICs.

2. Methods

2.1. Aortic valves and sera

Human tricuspid aortic valves were obtained from ten patients undergoing valve replacement for severe calcific aortic stenosis; blood samples were collected from the same patients before surgery. As controls, ten aortic non calcified valves were obtained from patients undergoing aorta and aortic valve replacement for ascending aortic aneurysm and aortic regurgitation. Serum samples were collected from ten healthy control subjects and not from patients with ascending aortic aneurysms, because it has been previously demonstrated that TRAIL is involved in aortic aneurismal disease [24] Informed consent was obtained from each patient and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

2.2. Cell cultures

Valve leaflets derived from calcified and non calcified control aortic valves were freshly obtained at surgery time and rinsed in sterile cold saline to remove blood. VICs derived from calcified (C-VICs) and non calcificied (N-VICs) aortic valves were isolated by sequential collagenase digestion. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco Invitrogen, Milan, Italy), 0,199 mmol/L penicillin, 0.172 mmol/L streptomycin, and 2.27 μmol/L fungizone in a humidified atmosphere containing 5% CO_2. Cells from passage 2 to 4 were used in all experiments. Each experiment was repeated three times using C-VICs and N-VICs derived from three different patients and performed using cells cultured in basal medium and after osteogenic differentiation. To obtain differentiated C-VICs (Dif-C-VICs) and N-VICs (Dif-N-VICs), C-VICs and N-VICs, respectively, were seeded in six-well tissue-culture plates and at confluence, ascorbic acid (0.28 mmol/L) (Sigma Aldrich), β-glycerophosphate (10 mmol/L) (Sigma Aldrich) and dexametasone (10 nmol/L) (Sigma Aldrich) were added to the medium for 21 days to induce osteoblastic differentiation.

2.3. Immunohistochemistry

Valve leaflets obtained at surgery time were rinsed in sterile cold saline to remove blood. One leaflet of each valve was fixed in 4% buffered formalin and paraffin-embedded; leaflets from both calcified and non calcified aortic valves were decalcified in 10% formic acid for seven days. Transversal sections with a thickness of 3 μm were performed from the base to the free edge in the middle portion of the valve. Immunohistochemistry was performed using the avidin–biotin complex method (Vector Laboratories, Burlingame, USA) according to the manufacturer's instruction. After boiling in citrate buffer (pH 6.0) for antigen retrieval, sections were incubated in 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity, washed with phosphate-buffered saline (PBS), and incubated in buffered normal horse serum to prevent unspecific binding of antibodies. The following primary antibodies were used: rabbit monoclonal anti human TRAIL (Abcam, Cambridge, UK), mouse monoclonal anti human CD4 (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal anti human CD68 (Novocastra, Leica Microsystems, Wetzlar, Germany). Sections were incubated with the primary antibodies overnight at 4 °C (anti TRAIL and anti CD4) or for 1 h at 25 °C (anti CD68). After washing, an appropriate biotin-labeled secondary antibody was applied for 1 h at 25 °C, followed by an avidin–biotin-peroxidase conjugate for 30 min. As chromogen, 3-amino-9-ethylcarbazole was used yielding a red reaction product. Cell nuclei were counterstained with Meyer's hematoxylin. The photomicrographs were obtained using a Nikon Eclipse E400 microscope equipped with a Nikon plan Apo 20x/0.75 DICM. The microscope was connected to a Nikon digital camera (DXM 1200); the acquisition was accomplished using the software LUCIA G version 4.61 (BUILD 0.64) for Nikon Italy.

2.4. Confocal microscopy

For cell characterization C-VICs and N-VICs from passage 1 were plated on coverslips (Sigma) and fixed in 3.7% paraformaldehyde. Fixed cells were washed three times with PBS, blocked in 1% BSA and 1% fetal bovine serum in PBS for 1 h. Cells were incubated with the following primary antibodies: mouse anti vimentin, mouse anti smooth muscle actin, and rabbit anti eNOS (Santa Cruz Biotechnology, Santa Cruz, CA). After washing, bound antibodies were detected using fluorescent-labeled goat anti-mouse or anti-rabbit F(ab′) fragment secondary antibody, Alexa Fluor 488, or Alexa Fluor 555 (Life Technologies). Nuclei were counterstained with TO-PRO-3 Iodide (Life Technologies).

Paraffin embedded tissue sections were deparaffinized in xylene and rehydrated in a series of graded alcohols to distilled water. Antigen retrieval was performed in citrate buffer (pH 6.0) using 750-W microwave for 5 min for three times. Sections were incubated in 3% hydrogen peroxide in methanol to block endogenous peroxidase activity, washed with phosphate-buffered saline, and incubated in buffer containing 2% bovine serum albumin to prevent unspecific binding of antibodies for 1 h at room temperature (RT). Double-label immunostaining was performed using primary rabbit monoclonal anti human TRAIL (Abcam) and mouse monoclonal anti human CD4 (Santa Cruz Biotechnology, Santa Cruz, CA) or mouse monoclonal anti human CD68 (Novocastra, Leica Microsystems, Wetzlar, Germany). The sections were incubated for 1 h at 37 ° C; after washing with PBS bound antibodies were detected using fluorescent-labeled goat anti-mouse or anti-rabbit F(ab′) fragment secondary antibody, Alexa Fluor 488, or Alexa Fluor 555 (Life Technologies) for 1 h at RT. Nuclei were counterstained with TO-PRO-3 Iodide for 10 min at RT (Life Technologies).

Stained C-VIC, N-VICs and tissue sections were then visualized and photographed by laser confocal microscopy TCS SP5 (Leica Mycrosystems, Mannheim, Germany). Images (2048 × 2048 pixels) were acquired with an oil immersion objective (X63 1.4 HCX PL APO; Leica Mycrosystems). Overlay images were assembled.

2.5. RNA isolation and real-time polymerase chain reaction (RT-PCR) amplification

C-VICs, N-VICs, Dif-C-VICs and Dif-N-VICs were subjected to mRNA extraction to detect the genic expression of DR4, DR5, DcR1, DcR2, osteocalcin (OC) and Runx2, using spin columns (Rneasy; Quiagen, Hilden, Germany) according to the manufacturer's instructions. RNA (1 μg) was reverse-transcribed, using the Super Script First-Stand Synthesis System kit for RT-PCR (Invitrogen, Carlsbard, CA, USA) 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 Inc., CA, USA). 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 [25]. The sense and antisense primers sequences of DR4, DR5, DcR1, DcR2, Runx2, glyceraldehyde phosphate dehydrogenase (GAPDH) (Operon) and OC (Sigma) are reported in Table 1.

2.6. Western blot analysis

To study the expression of TRAIL receptors, C-VICs, N-VICs, DIF-C-VICs and Dif-N-VICs 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-3 activation, C-VICs were seeded in 6-well tissue-culture plates and cultured with DMEM supplemented with 10% FBS. At confluence cells were starved with DMEM supplemented with 0.5% BSA for 12 h and stimulated with rh-TRAIL (Peprotech, Rocky Hill, NJ) 2.6 nmol/L. Cells were lysed at 0, 1, 2, 4, 6 and 8 h by incubation on ice for 30 min in lysis buffer containing 20 mmol Tris–HCl (pH 7.5), 1% Triton X- 100, 150 mM NaCl, 10% glycerol, 1 mmol Na_3VO_4, 50 mmol NaF, 100 mmol phenylmethylsulfonyl fluoride, and a commercial protease inhibitor mixture (Sigma Aldrich).

Cell proteins (30 μg) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to nitrocellulose membranes (Hybond; Amersham Pharmacia, London, UK). The blots were probed overnight at 4 °C with the primary antibody rabbit anti-DR4, anti-DR5, anti-DcR1, anti-DcR2 (Abcam, Cambridge, UK), anti Caspase-3 (Cell Signaling Technology, Inc., Danvers, MA) and

Table 1
Sense and antisense primer sequences.

Gene	Sense primer	Antisense primer
RT-h-DCR1	AAGTTCGTCGTCGTCATCG	GTGCCTCTGTTGCTGTGG
RT-h-DcR2	AAGTTCGTCGTCTTCATC	TTACAGGCTCCAGTATATTC
RT-h-DR4	CATCGGCTCAGGTTGTGG	GTTGCTCAGAATCGTTGTG
RT-h-DR5	GTGTCAGTGCGAAGAAGG	GCCACAATCAAGACTACGG
RT-h-OC	ACACTCCTCGCCCTATTG	CAGCCATTGATACAGGTAGC
RT-h-Runx2	GGAATGCCTCTGCTGTTATG	TTCTGTCTGTGCCTTCTGG
RT-h-GAPDH	TCATCCCTGCCTCTACTGGCG	TGCTTCACCACCTTCTTG

mouse anti β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). 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 Biotechnology Lincoln, Nebraska USA). Densitometric analysis was performed using LI-COR's Odyssey Infrared Imaging System Application Software version 3.0 (LI-COR Biosciences-Biotechnology).

2.7. Cell viability assay

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. C-VICs and N-VICs were cultured with DMEM supplemented with 10% FBS in 96-well tissue-culture plates. At confluence cells were stimulated with rh-TRAIL (Peprotech, Rocky Hill, NJ) (0, 2.6, 5.2 nmol/L) for 24 h. In parallel C-VICs were cultured with DMEM supplemented with ascorbic acid (0.28 mmol/L), β-glycerophosphate (10 mmol/L) and dexamethasone for 21 days to induce osteogenic differentiation. After 21 days rh-TRAIL (0, 2.6, 5.2 nmol/L) were added to the mineralizing cells for 24 h. MTT (Sigma Aldrich) 1.2 mmol/L was added to the culture media, followed by 4 h of incubation at 37 °C in a humidified 5% CO_2 atmosphere; the reaction was stopped by the addition of 150 μL 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, Hercules, CA). The results were normalized to cells incubated under control conditions.

2.8. Mineralized matrix nodules production

C-VICs were cultured in osteogenic medium to verify their ability to produce mineralized matrix nodules. Cells were seeded in 24-wells tissue-culture plates and cultured with D-MEM supplemented with ascorbic acid (0.28 mmol/L), β-glycerophosphate (10 mmol/L) and dexametasone (10 nmol/L); in parallel C-VICs were cultured in osteogenic medium added with TRAIL (Peprotech, Rocky Hill, NJ) 2.6 nmol/L. The medium was changed every 3 days and Von Kossa staining was performed after 21 days to identify osteoblast-differentiated cells able to produce mineralized nodules. The cells were fixed with 3% para-formaldehyde for 10 min, stained with silver nitrate (AgNO3), rinsed with distilled water, exposed for 1 h to bright light, and finally observed. The photomicrographs of mineralized nodules formed in cultures were obtained using a Nikon Eclipse E400 microscope equipped with a Nikon plan fluor 10x/030 DICL. The microscope was connected to a Nikon digital camera (DXM 1200); the acquisition and the quantitative analysis of the mineralized surface were accomplished by using the software LUCIA G version 4.61 (BUILD 0.64) for Nikon Italy. The quantification of mineralized nodules, as percentage of the total cell culture area, was represented by the histogram.

2.9. Elisa assay

Blood samples were collected in ten patients with calcific aortic stenosis scheduled for aortic valve surgery before the intervention and in ten normal control subjects. Serum levels of TRAIL were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The absorption was determined with an ELISA reader at 450 nm (550 Microplate Reader; Bio-Rad) and the results were expressed as mean ± SD.

2.10. 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). The results were considered statistically significant for *p* values less than 0.05.

3. Results

3.1. High TRAIL levels in valves and sera from patients with calcific aortic stenosis

The immunohistochemistry performed on ten human calcified aortic valves removed at cardiac surgery revealed that all calcified leaflets were strongly positive for TRAIL (Fig. 1A). On the contrary non calcified control human aortic valve leaflets showed no TRAIL immuno-positivity (Fig. 1B). Additionally calcified aortic valves (Fig. 1C, E) showed an inflammatory infiltrate that was not present in normal valves (Fig. 1D, F). Inflammatory cells were represented essentially by T lymphocytes, identified as $CD4^+$ cells (Fig. 1C), and macrophages, identified as $CD68^+$ cells (Fig. 1E). In order to understand the cellular source of TRAIL, we stained the tissue section simultaneously for TRAIL and CD4 or CD68. Confocal microscopy showed that TRAIL was co-expressed on $CD4^+$ and $CD68^+$ cells in calcific aortic valves (Fig. 1G, H), however not all CD4+ or CD68+ cells expressed TRAIL. According to the high expression of TRAIL in the calcific valves, we also demonstrated that serum levels of TRAIL were significantly higher in patients with calcific aortic stenosis (4 ± 1.54 pmol/L; range 2.5–7.1 pmol/L) compared to healthy control subjects (2.9 ± 0.77 pmol/L; range 1.8–4.1 pmol/L) ($p < 0.05$).

Thus, the high levels of TRAIL in calcific aortic valves are co-expressed on $CD4^+$ and $CD68^+$ cells; serum levels of TRAIL are significantly higher in patients with calcific aortic valve stenosis.

3.2. VICs express TRAIL receptors

Since in the calcified valves we demonstrated the expression of TRAIL, as next step we isolated N-VICs and C-VICs to study the genic and protein expression of TRAIL receptors. We showed that C-VICs expressed significantly higher mRNA levels of DR4, DR5, DcR1 and DcR2 compared with N-VICs (fold change 6.31, 2.35, 3.29 and 7.82 respectively; $p < 0.05$) (Fig. 2A). These results were also confirmed by Western blot analysis (Fig. 3). The dissimilar expression of TRAIL receptors in N-VICs and C-VICs is associated to a different phenotype of the two cellular populations. In particular, confocal microscopy showed that C-VICs intensively stained for vimentin and alpha smooth muscle actin, while N-VICs stained positively for vimentin and weakly for alpha smooth muscle actin (Fig. 4). Additionally, we found that C-VICs expressed significantly higher mRNA levels of Runx2 compared with N-VICs (fold change 2.77; $p < 0.05$) and similar levels of OC (Fig. 2B). We also studied the genic and protein expression of TRAIL receptors after 21 days of culture in osteogenic medium and found that both Dif-N-VICs and Dif-C-VICs expressed significantly higher mRNA and protein levels of DR4 (fold change 2.44 and 2.15 respectively; $p < 0.05$) compared to baseline (Figs. 2C and 3). Additionally after osteogenic differentiation both Dif-N-VICs and Dif-C-VICs expressed significantly higher mRNA levels of Runx2 (fold change 4.68 and 3.87 respectively; $p < 0.05$) and OC (fold change 2.23 and 3.57 respectively; $p < 0.05$) compared to baseline (Fig. 2D).

Thus, VICs derived from calcific valves express significantly higher mRNA and protein levels of DR4, DR5, DcR1 and DcR2 compared with VICs derived from non calcific valves; after osteogenic differentiation both cells express significantly higher mRNA and protein levels of DR4.

3.3. VIC sensitivity to TRAIL-induced apoptosis

The findings that VICs expressed TRAIL receptors, prompted us to evaluate their sensitivity to TRAIL apoptotic effect. Both undifferentiated (N-VICs and C-VICs) and differentiated (Dif-N-VICs and Dif-C-VICs) cells were treated with increasing concentrations of rh-TRAIL (ranging from 0 to 5.2 nmol/L) and cell viability was determined after 24 h. The lowest concentration of the cytokine able to reduce significantly the cell viability in both C-VICs and Dif-C-VICs was 2.6 nmol/L, however the former were more sensitive to TRAIL apoptotic effect than the latter. In particular, in C-VICs treated with 2.6 nmol/L TRAIL the cell viability decreased about 30% ($p < 0.01$), whereas in Dif-C-VICs of about 15% ($p < 0.05$). At 5.2 nmol/L TRAIL C-VICs did not show a further reduction of cell viability, while in Dif-C-VICs we found an additional decline until 29% ($p < 0.01$), thus reaching the same percentage (Fig. 5A). N-VICs and Dif-N-VICs had a similar trend of C-VICs and Dif-C-VICs (data not shown). To further validate the results obtained from MTT assay, we evaluated the activation of the apoptotic cascade induced by TRAIL, with particular regard to the involvement of caspase-3, the main effector caspase of apoptotic cell death. This enzyme is normally present in the cells as an inactive zymogen form (pro-caspase-3, p35) and requires proteolytic processing before it becomes active, p17. Thus, we examined whether the cleavage of caspase-3 could be induced by TRAIL treatment in C-VICs. As shown by western blot analyses in Fig. 5B, the p17 cleaved form of caspase-3 was detected in C-VICs at 4, 6 and 8 h after treatment with 2.6 nmol/L of rh-TRAIL.

TRAIL reduced cell viability of VICs derived from both calcific and non calcific valves and activated the apoptotic cascade.

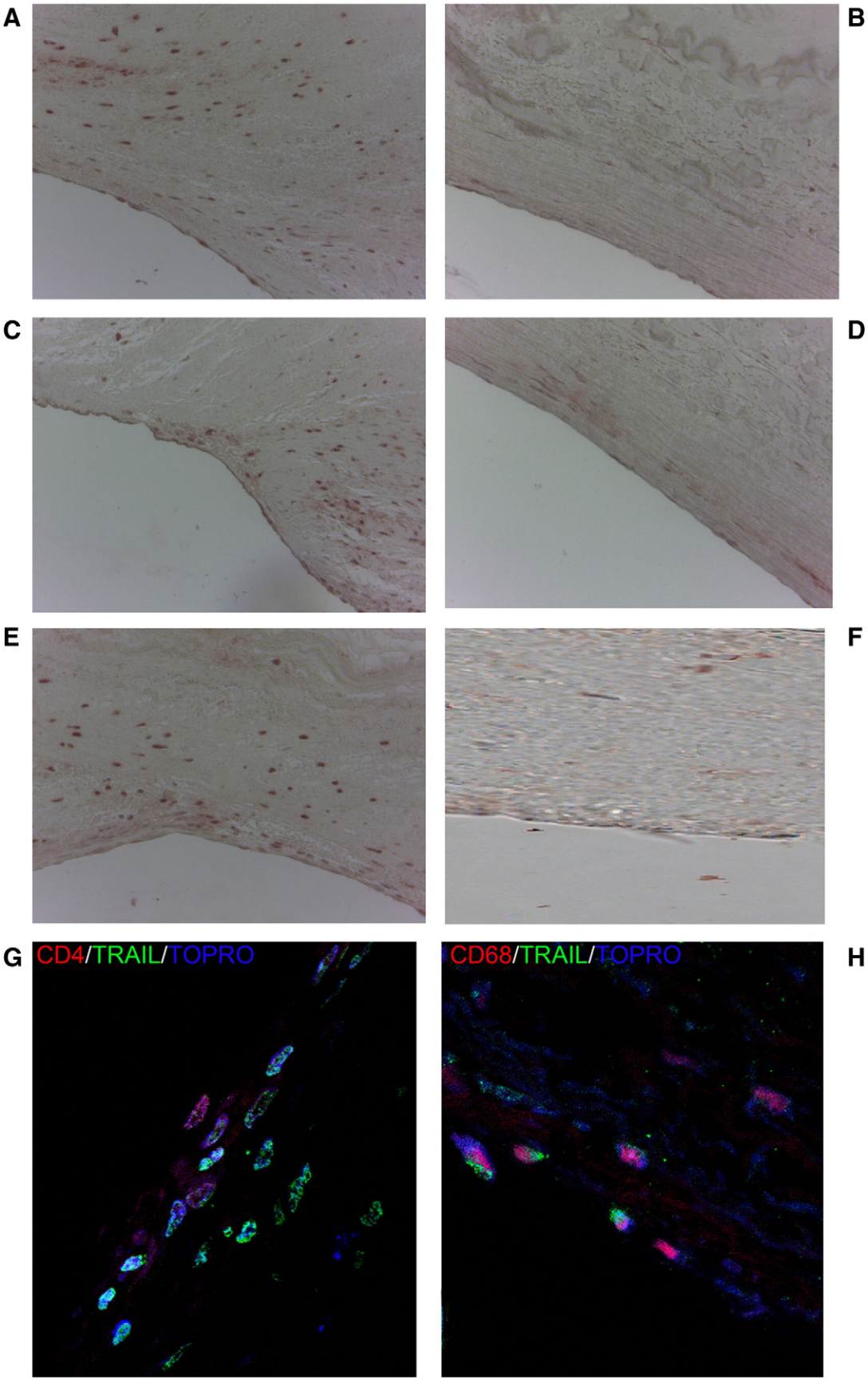

Fig. 1. The presence of TRAIL in human calcified aortic valves and normal aortic valves. Histological sections showing positive immunostaining for TRAIL (A), CD4 (C) and CD68 (E) in calcific aortic valves. Normal aortic valves show no staining for TRAIL (B), CD4 (D) and CD68 (F). (Original magnification 20×). Confocal micrographic images showing coimmunostaining of TRAIL (green) and CD4 (red) (G) or CD68 (red) (H) in calcific aortic valves. Nuclei were counterstained with TO-PRO-3 iodide (blue).

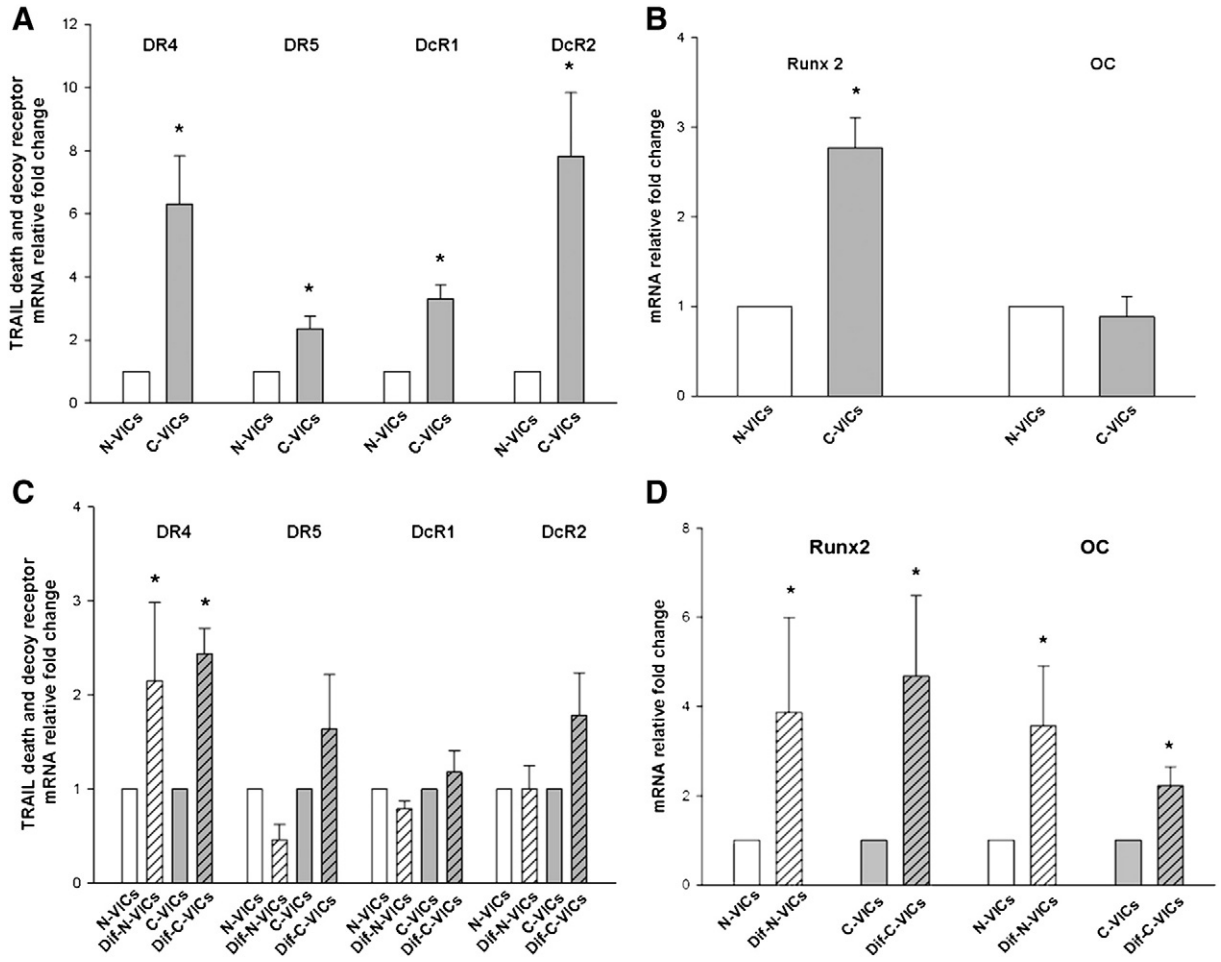

Fig. 2. Expression of TRAIL receptors, Runx2 and Osteoclacin in N-VICs, C-VICs, Dif-N-VICs and Dif-C-VICs. A: mRNA levels of DR4, DR5, DcR1, DcR2 in N-VICs and C-VICs were quantified using RT-PCR and data are presented relative to the gene expression of GAPDH (mean ± SE); * $p < 0.05$. B: mRNA levels of Runx2 and osteocalcin (OC) in N-VICs and C-VICs were quantified using RT-PCR and data are presented relative to the gene expression of GAPDH (mean ± SE); * $p < 0.05$. C: The gene expression of DR4, DR5, DcR1, DcR2 in N-VICs, Dif-N-VICs, C-VICs and Dif-C-VICs was evaluated using RT-PCR and data are presented relative to the gene expression of GAPDH (mean ± SE); * $p < 0.05$. D: The gene expression of Runx2 and osteocalcin (OC) in N-VICs, Dif-N-VICs, C-VICs and Dif-N-VICs was evaluated using RT-PCR and data are presented relative to the gene expression of GAPDH (mean ± SE); * $p < 0.05$.

3.4. TRAIL enhances the mineralized nodule formation

We assessed whether TRAIL might promote calcification of VICs and evaluated the synthesis of mineralized matrix nodules by the C-VICs in the presence or absence of TRAIL. The Von Kossa staining showed that C-VICs cultured in osteogenic medium for 21 days are able to produce matrix mineralized nodules. The addiction of 2.6 nmol/L rh-TRAIL to the osteogenic medium enhanced the production of the nodules (Fig. 6A). Additionally we evaluated the effect of TRAIL treatment on the genic expression of Runx2 and TRAIL receptors in C-VICs. RT-PCR results showed that the addiction of rh-TRAIL 2.6 nmol/L to the basal medium did not affect Runx2 expression in C-VICs, while culturing the cells in osteogenic medium in the presence or absence of TRAIL induced a significantly higher expression of mRNA levels of Runx2 (fold change 4.33 and 4.99, respectively; $p < 0{,}05$) (Fig. 6B). Moreover, we found that the addiction of TRAIL to the basal and the osteogenic medium induced a significantly higher expression of DR4 mRNA levels (fold change 2.61 and 3.11 respectively; $p < 0.05$) compared to the basal conditions; mRNA levels of DR5, DcR1 and DcR2 were not modified by TRAIL treatment (Fig. 6C). These results were also confirmed by Western blot analysis, DR4 modulation was shown in Fig. 6D.

Thus, TRAIL enhanced the formation of mineralized nodules in VICs derived from calcific aortic valves cultured in osteogenic medium.

4. Discussion

In this study we firstly demonstrated that the pro-apoptotic cytokine TRAIL is expressed in human calcified aortic valves but not in normal ones. The presence of pro-apoptotic cytokines has been previously reported in human calcified aortic valves [6] but to our knowledge the presence of TRAIL has been detected only in CD4 T cells of atherosclerotic lesions [26,27]. Its expression in calcific aortic valves is associated with an inflammatory infiltrate represented essentially by T cells and macrophages that is not present in normal aortic valves. In fact, TRAIL can be expressed by various activated cells of the immune system such as natural killer cells, T cells, dendritic cells and macrophages. According to previous reports attesting the presence of T cells and macrophages in calcific aortic valves [9,28,29] as well as their ability to produce cytokines, our results demonstrated that the main source of TRAIL in calcific aortic valves is represented by the inflammatory infiltrate. According to the high expression of TRAIL in the calcific valves, we showed that serum levels of TRAIL are significantly elevated in patients with CAVD compared to normal subjects. These data are consistent with previous studies reporting that TRAIL serum levels are elevated in patients with inflammatory, immune and apoptosis-mediated diseases including systemic lupus erythematosus [30], psoriatic arthritis [31], diabetes and diabetic nephropathy [32], primary biliary cirrhosis [33],

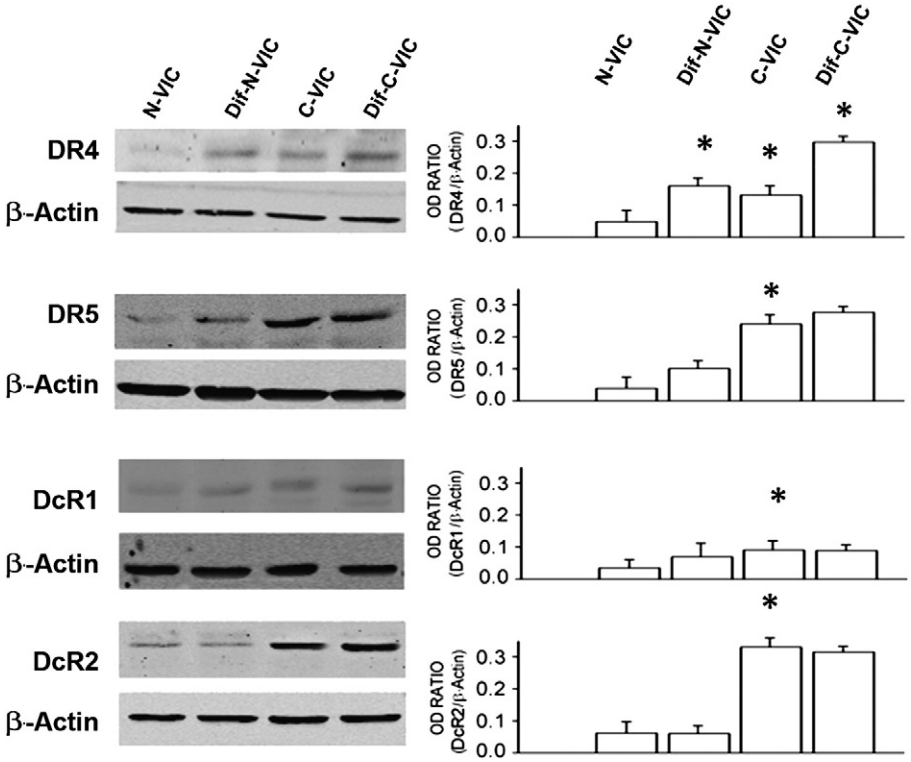

Fig. 3. Protein expression of DR4, DR5, DcR1, DcR2 in N-VICs, Dif-N-VICs, C-VICs and Dif-C-VICs by Western blot analysis; the intensity of the bands obtained by Western blot was quantified by densitometry (histogram) and normalized to β-actin.

allogenic transplant rejection [34] cardiomyopathy [35], myeloma and periodontitis [36]. Additionally, we demonstrated that both VICs derived from normal and calcific aortic valves express all TRAIL receptors; in particular, C-VICs show significantly higher levels of DR4, DR5, DcR1 and DcR2 compared to N-VICs. It is known from literature that VICs represent a heterogeneous cellular population deputed to the maintaining of the structural integrity of the valve and extracellular matrix remodeling through protein synthesis and enzymatic degradation operated by matrix metalloproteinases [37]. VIC phenotype is considered plastic and reversible: most VICs in normal valves are quiescent (qVIC) with a fibroblast-like phenotype, whereas VICs in developing, adapting and diseased valves are activated (aVIC) and assume a myofibroblast phenotype with the expression of contractile proteins [38]. In pathological conditions VICs can even acquire an osteoblast-like phenotype (ob-VICs) associated to the expression of osteogenic protein as OPN, bone sialoprotein, alkaline phosphatase, and BMP-2 and -4 [12,16,17,39] as well as transcription factors as Runx2, involved in developmental processes such as valvulogenesis and osteogenesis [40]. Our results confirm the phenotypic plasticity of VICs. N-VICs were phenotypically fibroblasts whereas C-VICs expressed contractile proteins; additionally C-VICs express significantly higher levels of Runx2 compared to N-VICs. Interestingly, we found that the transition from a fibroblast to a myofibroblastic phenotype (N-VICs versus C-VICs) is also associated with a higher expression of all TRAIL receptors. Additionally, the osteoblast-like phenotype obtained after 21 days of VIC osteogenic differentiation is characterized by a higher expression of Runx2 and OC,

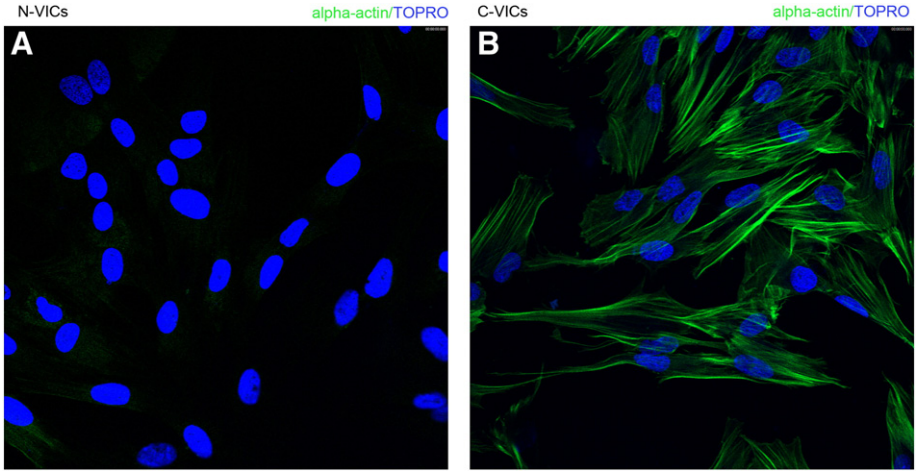

Fig. 4. Confocal micrographic images showing immunostaining for alpha-actin (green) in N-VICs (A) and C-VICs (B). Nuclei were counterstained with TO-PRO-3 iodide (blue).

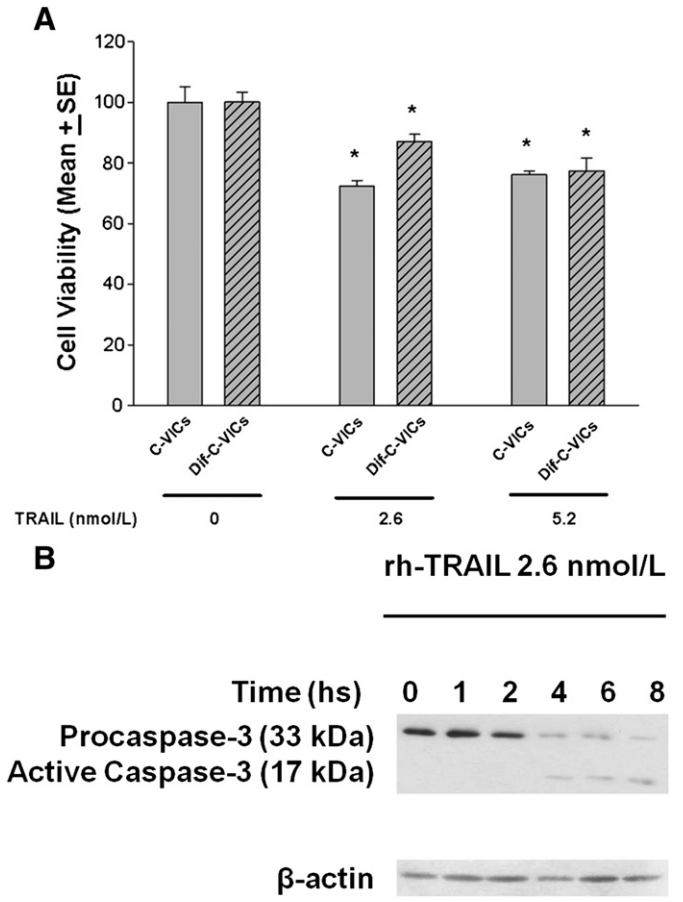

Fig. 5. Effect of TRAIL on cell viability and caspase-3 activation. A: C-VICs and Dif-C-VICs treated for 24 h with TRAIL at the indicated concentrations were subjected to MTT assay to evaluate cell viability. Results are expressed as mean values of optical density at 570 nm ± standard error (SE). Four independent experiments were performed and each treatment was done in quadruplicate; * $p < 0.05$. B: C-VICs, treated with 2.6 nmol/L TRAIL, were lysed at the indicated times and analysed by western blot analysis to detect the protein levels of the cleaved form of caspase-3 (fragment at 17 kDa). β-actin was utilized as control. The figure shows one of four independent experiments.

together with the increase of DR4 levels. The expression of TRAIL receptors in human VICs is associated with the sensitivity to TRAIL-mediated apoptosis involving caspase-3 activation, that in N-VIC and C-VIC is associated to the increased expression of DR4 and DR5, whereas in Dif-N-VIC and Dif-C-VIC of DR4. Our data overlap literature data demonstrating that TRAIL not only induces cell death in a wide variety of tumor cell lines, but also in various normal cells, including vascular smooth muscle cells (VSMCs) [27] and bone cells [41,42]. VICs sensitivity to TRAIL apoptotic effect is of paramount importance because apoptosis has been shown to be an initiator of vascular calcification in *in vitro* studies [43]; in particular Proudfoot and coll. demonstrated that increased apoptosis precedes calcification in VSMC cultures and that the apoptotic bodies

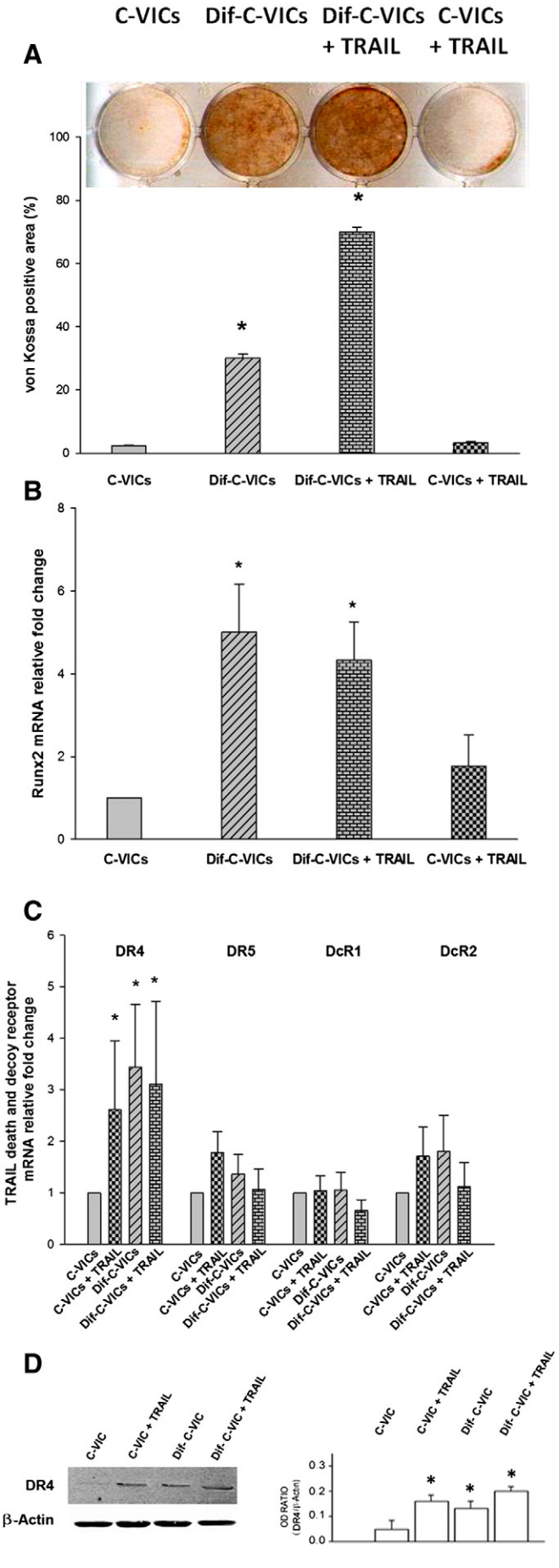

Fig. 6. TRAIL enhanced the mineralized nodule formation. A: Von Kossa staining in C-VICs cultured in an osteogenic medium with and without TRAIL treatment; the von Kossa positive area was quantified relative to the total culture area. B: gene expression of Runx2 in C-VICs cultured in basal and osteogenic medium with and without TRAIL treatment; mRNA levels were quantified using RT-PCR and data are presented relative to the gene expression of GAPDH. C: gene expression of DR4, DR5, DcR1, DcR2 and Runx2 in C-VICs cultured in basal and osteogenic medium with and without TRAIL treatment; mRNA levels were quantified using RT-PCR and data are presented relative to the gene expression of GAPDH. Data are shown as mean ± SE; * $p < 0.05$. D: Protein expression of DR4 in C-VICs cultured in basal and osteogenic medium with and without TRAIL treatment by western blot analysis; the intensity of the bands obtained by Western blot was quantified by densitometry (histogram) and normalized to β-actin.

may act as nucleating structures for calcium crystal formation [43]. Currently only few works focused on the role of apoptosis in the pathogenesis of CAVD [6,44,45]. Jian et al. showed that the TGF-β1 is present in human calcific aortic stenotic cups and promotes calcification of cultured sheep aortic valvular interstitial cells (SAVICs) through mechanisms involving apoptosis [6]; in fact the administration of an apoptosis inhibitor to SAVICs cultured in an osteogenic environment results in a significant decrease in nodules calcification, thereby demonstrating that a certain level of apoptosis is necessary for the calcification of nodules in these cultures [6]. Extracellular-regulated kynases 1 and 2 (ERK1/2) regulate proliferation, differentiation and apoptosis in various cell lines [46]. In vitro administration of an ERK pathway inhibitor significantly decreased apoptosis and nodules calcification in cultured porcine aortic VICs enabling these cells to retain a quiescent phenotype [45]. Our *in vitro* experiments firstly showed that the sensitivity to TRAIL-mediated apoptosis together with the induction of the calcification process in human VICs cultured in osteogenic medium leads to a significant increase of mineralized matrix nodule deposition. In this process, it seems that DR4 has a prominent role, because its expression is strongly up-regulated by the osteogenic induction, whereas the expression of the other TRAIL receptors does not change significantly.

5. Conclusions

The present study supports the concept that TRAIL may contribute to the pathogenesis of CAVD by initiating the apoptosis associated mineralization of VICs. Our results indicate that the secretion of TRAIL by leukocytes infiltrating the aortic cusps may induce VIC apoptosis and that apoptotic bodies may act as nuclear site for calcium deposition. Thus, TRAIL may be a novel regulator of extracellular matrix remodeling in aortic valve calcification and a potential new target in the therapeutic management of patients with CAVD.

Disclosures

This investigation was supported by Agenzia Spaziale Italiana (ASI-OSMA grant to Maria Grano) and Ministero dell'Istruzione Università e Ricerca (ex 60% to Maria Grano).

Acknowledgments

The authors wish to thank for support on immunohistochemistry Mrs Michela De Giorgis (Department of Basic Medical Sciences, Neurosciences and Sense Organs, Section of Human Anatomy and Histology, University of Bari, Italy) and Dr Andrea Marzullo (Department of Emergencies and Organ Transplantation, Section of Pathological Anatomy, University of Bari, Italy).

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