Increased OPG Expression and Impaired OPG/TRAIL Ratio in the Aorta of Diabetic Rats

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Abstract: Despite accumulating evidence showing that TNF-related apoptosis inducing ligand (TRAIL) plays a role in vascular biology and that its decoy receptor osteoprotegerin (OPG) is expressed in the vessel wall, modulation of these TNF and TNF-R family members in the early phases of diabetes mellitus has not been investigated. The expression of TRAIL and of OPG was examined both at the mRNA and protein levels in control and streptozotocin (SZT)-induced diabetic rats at early time points after the induction of diabetes mellitus. No differences in the steady-state mRNA levels of TRAIL were noticed by quantitative RT-PCR among the two groups of animals. On the other hand, diabetic rats showed a rapid and significant increase of the steady-state mRNA levels of OPG in the aortic wall of diabetic animals with respect to vehicle-treated (control) animals. These findings were confirmed at the protein level by analysing the amount of TRAIL and OPG proteins in aortic lysates by either Western blot or immunohistochemistry. Thus, an abnormal elevation of the OPG/TRAIL ratio in the vessel wall characterizes the early onset of diabetes mellitus and might represent a molecular mechanism involved in the vascular dysfunction characterizing diabetes mellitus.

Key words: TRAIL, OPG, diabetes mellitus, aortas, rats.

INTRODUCTION

TNF-related apoptosis inducing ligand (TRAIL) is member of the tumour necrosis factor (TNF) family of cytokines, which exists either as a type II membrane or as a soluble protein [1]. TRAIL interacts with four high-affinity transmembrane receptors (TRAIL-R1, -R2, -R3 and -R4), and with a soluble decoy receptor, osteoprotegerin (OPG), all belonging to the apoptosis-inducing TNF-receptor (R) family [1]. In spite of its established role as a mediator of anticancer activity of the immune system [2, 3], the wide expression of TRAIL and TRAIL receptors in many normal tissues, including the vascular system [4-7], suggests that TRAIL likely has additional biological functions. In this respect, it has been shown that OPG is produced by a wide range of tissues, including the cardiovascular system, and that OPG levels are particularly high in aortic and renal arteries [7, 8]. Moreover, vascular smooth muscle cells (VSMCs) constitutively express OPG, and its release in the culture supernatants is significantly increased *in vitro* by inflammatory cytokines [8]. Other studies have reported that serum OPG is significantly increased in diabetic patients [9, 10], and in patients affected by coronary artery disease, myocardial infarction and abdominal aortic aneurysm [11-13].

Endothelial cell dysfunction is an important risk factor in the pathogenesis of atherosclerosis, whose prevalence is markedly increased among individuals affected by diabetes mellitus [14]. In this respect, it has been previously demonstrated that TRAIL induces the *in vitro* release of nitric oxide (NO) by both hematopoietic cells and vascular endothelial cells [15-16]. Moreover, TRAIL significantly counteracts the pro-adhesive activity of inflammatory cytokines on endothelial cells *in vitro* [17] and shows anti-atherosclerotic activity *in vivo* [18]. On these bases, we have here investigated expression level of both TRAIL and OPG in the aortic walls obtained from normal and diabetic rats.

MATERIALS AND METHODS

Animals and Tissues

Diabetes mellitus was induced in 3-month-old male Wistar rats by injecting 40 mg/kg streptozotocin (SZT, Sigma Chemicals, St Louis, MO) freshly dissolved in 0.1 M citrate buffer pH 4.5 into the femoral vein under pentobarbital anaesthesia. Non-diabetic age-matched control rats received vehicle infusion *via* the same vein. All animal procedures were approved by the Animal Care Committee of the University of Trieste. Non-fasting blood glucose concentrations and body weight of SZT-diabetic rats (SZT, n=15) and age-matched control non-diabetic rats treated with vehicle (n=5) were measured at day 5 and 15, when animals were sacrificed with CO_2. The initial body weight of diabetic rats was 254 ± 8 g, while the final body weight was 256 ± 23 g. The initial body weight of diabetic rats treated with insulin was 248 ± 12 g, while the final body weight was 266 ± 18 g. On the other hand, the initial body weight of non-diabetic rats was 249 ± 9 g, while the final body weight was 284 ± 27 g. Blood glucose was measured using an AccuCheck glucose monitor (Roche, Indianapolis, IN). After sacrifice of the animals, aortas were harvested, cleaned of connective tissue and used for analytical determinations.

RNA Extraction and RT-PCR Amplification

Immediately after cleaning, approximately 20 mg of aortic tissue was stabilized in RNAlater (Qiagen, Hilden, Ger-

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many). Tissues were grinded to a fine powder under liquid nitrogen and total RNA was extracted and isolated from samples by Qiagen RNeasy Fibrous Tissue kit (Qiagen) following manufacturer's instruction. First strand cDNA was synthesized from 1 μg of purified total RNA by incubating with 2.5 U/μl of Moloney murine leukemia virus reverse transcriptase in a 20 μl reaction mixture containing 1 mM of each dNTP and 2.5 μM of random hexamers, as described in Perkin Elmer RNA PCR Core kit instructions, and incubated for 60 minutes at 42°C followed by 5 minutes at 99°C.

SYBR green real time PCR assay was performed by Light-Cycler technology (Roche) in 20 μl PCR mixture volume consisting of 10μl of 2X Quantitect SYBR Green PCR Master Mix (Qiagen), containing HotStarTaq DNA polymerase, 500 nM of each oligonucleotide primer and 5μl of retrotranscribed cDNA. SYBR green real time PCR was carried out using these specific oligonucleotide pairs: rat TRAIL (5'-GCACTTGAGAAACGGAGAGC-3' and 5'-CTGGCACTC TTCATCAGCAG-3'), rat OPG (5'-GAAAGCACCCTGTA GGAAACAC-3' and 5'-TAATCTTGGTAGGCACAGCAA A-3') and rat GAPDH (5'-ATCACTGCCACTCAGAAGA C-3' and 5'-ACATTGGGGGTAGGAACAC-3'). The quantitation of each mRNA was performed by reference specific curve as described by Kim H *et al*. [19]. Quantitation is expressed as specific OPG or TRAIL mRNA copies number /10.000 GAPDH mRNA copies.

Western Blot Analysis

Aortic rings were homogenized with a tissue grinder (Wheaton, Melville, NJ) in PBS containing 2% NP-40, 0.5% sodium deoxycolate, 0.1% SDS at 4°C. Protein determination was performed by Bradford assay (Bio-Rad, Richmond, CA). Proteins (50 μg) were migrated in acrylamide gels, blotted onto nitrocellulose filters and then processed following the detection protocol of Lumi-Light Plus Western Blotting Substrate (Roche). For Western blot analysis, anti-TRAIL mouse IgG (final dilution 1:1000, R&D Systems, Minneapolis, MN), anti-OPG goat polyclonal antibody (Ab, final dilution 1:200, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-actin mouse IgM (reacting with α, β and γ isoforms, final dilution 1:10000, Oncogene, Boston, MA) were used. The secondary Abs employed were respectively anti-mouse IgG (final dilution 1:2000) or anti-mouse IgM (final dilution 1:2000) or anti-goat IgG (final dilution 1:2000). All secondary Abs were peroxidase-conjugated (Sigma). Blotting densitometry was performed by using a Optimas software (Optimas Corporation, Washington, DC).

Immunohystochemical Analysis

Aortic specimens (typically taken from abdominal aortas) were fixed with 4% paraformaldehyde in phosphate buffer solution (PBS) at room temperature for 2 hours. After fixation, the specimens were processed for paraffin embedding. Sequential 10 μm thick sections were cut from each aorta specimen, mounted on slides and kept for 2-3 days at 37°C, to avoid their detachment. For OPG determination, aortic sections were deparaffinized and hydrated through xylene and graded alcohol series. To improve antigen recognition, slides were placed in 10 mM citrate buffer at 90°C and left in citrate buffer until they reached room temperature. After washing in PBS, background staining caused by endogenous peroxidase activity was quenched by pre-incubating aortic sections in a 0.3% H_2O_2 solution in H_2O for 30 minutes at room temperature. The specimens were next incubated for 30 minutes with normal blocking serum, which was prepared from the species in which the secondary Ab is made, and then with the primary Abs described above (anti-OPG, Santa Cruz, dilution 1:60) overnight at 4°C. Immunodetection was performed by Vectastain Universal *Elite* ABC Kit (Vector Laboratories, Burlingame, CA) following the manufacturer's instructions.

After immunostaining, images of aortic slides were acquired using a high-performance CCD CoolSNAP video camera system (Roper Scientific Inc., Tucson, AZ) mounted on an Axiophot Zeiss light microscope. Densitometry was performed on computerized images of aortic sections using the Optimas software (Optimas).

Statistical Analysis

The median, minimum, and maximum values were calculated for each group of data obtained from both human and mouse serum samples. Box plots were used to show the median, minimum and maximum values and 25th to 75th percentiles. Analysis of variance with Student's *t*-test was used to compare mean values. Differences were considered significant when $p < 0.05$.

RESULTS

The Expression of Aortic TRAIL is Similar in Diabetic and Control Rats

Diabetes mellitus was induced by destruction of pancreatic islet cells by treating rats with SZT (40 mg/kg). The loss of endogenous insulin secretion resulted in stable diabetes, defined as blood glucose levels >250 mg/dl, already after 7-10 days from STZ injection (Fig. (**1**)). Since we were interested in evaluating whether modifications in the TRAIL and/or OPG expression levels took place in the early events after diabetes mellitus induction, the two groups of animals were sacrificed at day 21 from the beginning of the experiment.

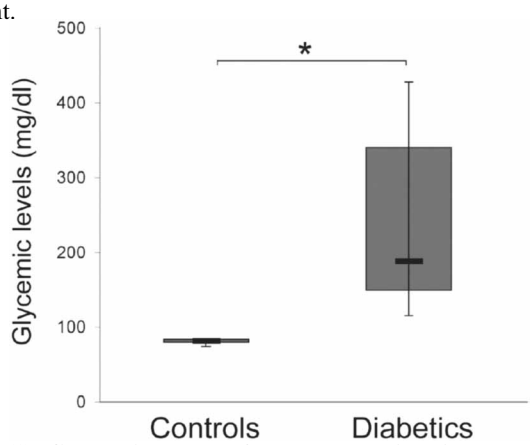

Fig. (1). Glycaemia values of control and diabetic rats. Blood glucose levels were evaluated after 7 days from diabetes mellitus induction by STZ-injections. Horizontal bars are medians, upper and lower edges of box are 75th and 25th percentiles, lines extending from box are 10th and 90th percentiles. *, $p < 0.05$.

SZT-induced diabetic rats did not show any significant difference in the steady-state mRNA levels of TRAIL with respect to untreated animals, as evaluated by quantitative RT-PCR analyses of aorta sections (Fig. (**2A**)). To validate the mRNA data at the protein level, TRAIL expression was analyzed by Western blot. The lack of differences in TRAIL protein expression between diabetic and control rats was quantitatively evaluated by densitometric analysis of all the aortic samples examined, and the results are shown together with representative Western blot results (Fig. (**2**)).

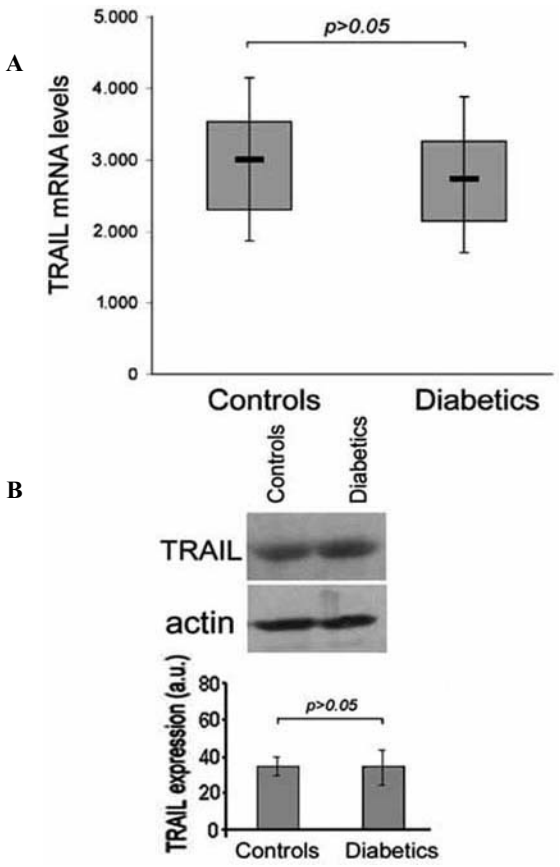

Fig. (2). Expression of TRAIL in rat aortas. Sections of aortas obtained from control and SZT-treated diabetic rats were analyzed for TRAIL expression. In **A**, sections of aortas were subjected to RNA extraction and the levels of TRAIL mRNA were analysed by quantitative RT-PCR and normalized respect to 10.000 copies of GAPDH. Horizontal bars are medians, upper and lower edges of box are 75th and 25th percentiles, lines extending from box are 10th and 90th percentiles. In **B**, protein lysates from aorta sections were analyzed for TRAIL protein levels by Western blot. Actin staining is performed as loading control. Representative samples are shown. Protein bands were quantified by densitometry and levels of TRAIL were calculated for each sample, after normalization to actin (a.u., arbitrary units). Data are expressed as means ± SD of 5 control and 10 diabetic rats, each analyzed in duplicate.

The Expression of Aortic OPG is Markedly Increased in Diabetic Rats

Although the best-characterized activity of OPG is to inhibit osteoclastic differentiation induced by RANKL, it has been recently shown that the major source of OPG in the body is the vessel wall [20], clearly suggesting that OPG plays an important role in vascular biology and physiopathology. We could confirm that the rat aortas obtained from normal rats showed significant steady-state mRNA levels of OPG (Fig. (**3A**)). However, at variance to TRAIL, whose mRNA levels did not vary between normal and diabetic rats, the OPG mRNA steady-state levels were significantly increased in SZT-treated diabetic rats with respect to control animals ($p<0.05$) (Fig. (**3A**)). Thus, the ratio between the steady-state mRNA levels of OPG and TRAIL in aortic walls was significantly ($p<0.05$) increased in diabetic versus normal rats (Fig. (**3B**)).

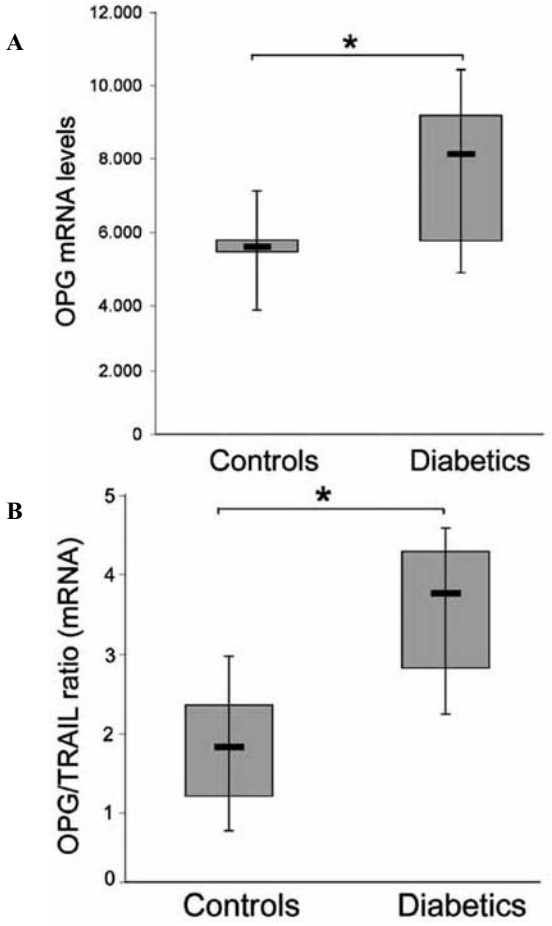

Fig. (3). Expression of OPG mRNA and OPG/TRAIL ratio in rat aortas. A, Sections of aortas obtained from control and SZT-treated diabetic rats were analyzed for OPG expression. In **A**, sections of aortas were subjected to RNA extraction and the levels of OPG mRNA were analysed by quantitative RT-PCR and normalized respect to 10.000 copies of GAPDH. In **B,** the OPG/TRAIL mRNA ratios were calculated in diabetic versus normal rats. Horizontal bars are median, upper and lower edges of box are 75th and 25th percentiles, lines extending from box are 10th and 90th percentiles. *, $p< 0.05$.

Although OPG is a secreted protein, no commercial kits are available to measure the serum levels of OPG as well as TRAIL in the rat sera (data not show), Nevertheless, we

were able to validate the mRNA data at the protein level, by analyzing the amount of OPG protein in the aortas of control and diabetic rats by Western blot analysis (Fig. (**4A**)). A significant ($p<0.05$) increase of the amount of OPG protein was observed in the aortas of diabetic rats with respect to control rats, as demonstrated by densitometric analysis of all aortic samples examined (Fig. (**4A**)). The increased expression of OPG protein was documented also by immuno-histochemistry (Fig. (**4B**)), which confirmed that OPG immunoreactivity was significantly increased in the aorta of diabetic rats.

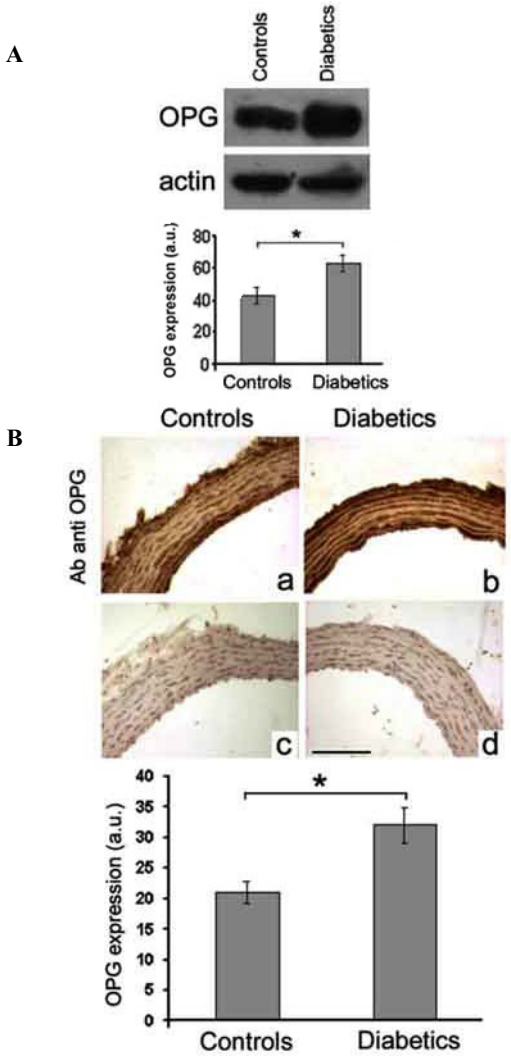

Fig. (4). Expression of OPG protein in rat aortas. In **A**, protein lysates from aorta sections were analyzed for OPG protein levels by Western blot. Actin staining is performed as loading control. Representative samples are shown. Protein bands were quantified by densitometry and levels of OPG were calculated for each sample, after normalization to actin (a.u., arbitrary units). **B** shows representative sections of rat aortas examined immunohistochemically by using an anti-OPG mAb followed by a secondary Ab (panels a, b) or with secondary Ab alone (panels c, d). The densitometric quantification of OPG immunostaining, expressed as arbitrary units (a.u.) is shown. Data are expressed as means ± SD of 5 control and 10 diabetic rats, each analyzed in duplicate.

DISCUSSION

Although it has been previously reported that the serum levels of OPG are significantly elevated in diabetic patients [9, 10], we have demonstrated for the first time that the early onset of diabetes mellitus is accompanied by a significant up-regulation of the expression of OPG in the aortic wall of diabetic rats with respect to vehicle-treated animals. On the other hand, the expression levels of TRAIL were unchanged in diabetic versus control animals. In this respect, although contrasting results have been reported on the affinity of TRAIL for OPG [21, 22], when a rationally designed small molecule mimic of OPG was examined for association with TRAIL in binding studies, this peptide, termed OP3-4, bound to TRAIL at a *Kd* of 1.93×10^{-5} M, showing an affinity for TRAIL similar to that of transmembrane TRAIL receptors [23].

There is now mounting evidence to suggest that the OPG/TRAIL interaction is biologically important at least in *in vitro* culture systems. OPG has been shown to act in a paracrine or autocrine manner by binding TRAIL and promoting the survival of prostate cancer cells [24], breast cancer cells [25] and multiple myeloma cells [26]. However, all these studies were performed at the cellular levels but the ratio of TRAIL and OPG relative expression has not been evaluated in *in vivo* models. On the other hand, although we were unable to evaluate the plasma levels of OPG in both normal and diabetic rats since the commercially available ELISA kits for human and mouse OPG do not cross-react with rat OPG (data not shown), we could demonstrate that an imbalanced TRAIL versus OPG expression is present in the local microenvironment of aortic walls. These findings are noteworthy taking into account of the protective effects of TRAIL in vascular physiopathology [16, 17]. The ability of TRAIL to show a blood-vessel-related protective effect is unique among the TNF family members and it is in sharp contrast with the biological effect of TNF-α, the prototype member of the TNF super-family of cytokines, which is a key mediator of vascular dysfunction in diabetes mellitus [14].

In conclusion, our present findings, together with the evidence that TRAIL displays anti-atherosclerotic activity in ApoE null mice [18], suggests that a therapeutic strategy aimed to decrease the expression/release of OPG and/or to increase the levels of TRAIL may be suitable for improving the vascular function in diabetes mellitus.

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ABBREVIATION

TRAIL = TNF-related apoptosis inducing ligand

OPG = Osteoprotegerin

SZT = Streptozotocin

NO = Nitric oxide

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