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Trail and vascular injury

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1. ABSTRACT

Cardiovascular diseases are the leading cause of mortality in the Western world. The underlying pathological process is a thickening of the arterial wall due to the formation of atheromatous plaques which contain a lipid core covered by a fibrous cap. The main mechanisms involved in atherogenesis are: lipoprotein retention, endothelial cell activation, vascular smooth muscle cell proliferation, macrophage infiltration, proteolytic injury, neovascularization and apoptosis. Different members of the tumor necrosis factor family (TNF) of proteins have been detected in human atherosclerotic plaques, among these are TNF-related apoptosis-inducing ligand (TRAIL) and its receptors (TRAIL-Rs and osteoprotegerin, OPG). In this review, the involvement of TRAIL and its receptors in the mechanisms underlying atherothrombosis is reviewed. In this respect, there are still some controversial data on the effects of TRAIL on inflammation and apoptosis of vascular cells. However, recent *in vivo* studies have suggested a potential proinflammatory and proapoptotic role of TRAIL in vascular injury. In addition, soluble forms of the TNF-superfamily can be released extracellularly and have been detected in human plasma. For this reason, we different studies evaluating the potential use of TRAIL and OPG plasma levels as markers of vascular injury are discussed.

2. ATHEROSCLEROSIS

Atherothrombosis is the leading cause of mortality in the Western world, clearly outnumbering deaths attributed to malignant or infectious diseases (1). The underlying pathological process is a thickening of the arterial wall owing to the formation of atheromatous plaques which contain a lipid core covered by a fibrous cap (2) (Figure 1). Atherosclerosis preferentially affects various regions of the circulation yielding distinct clinical manifestations depending on the particular circulatory bed affected. Atherothrombosis of the coronary arteries commonly causes myocardial infarction and angina pectoris. When the affected vessels are the arteries supplying the central nervous system it provokes stroke and transient cerebral ischemia. In the peripheral circulation, atherosclerosis causes intermitent claudication and gangrene (3).

The early phases of atherothrombosis are characterized by lipoprotein retention and interaction with both, resident cells (endothelial and smooth muscle cells, SMC) and infiltrating cells (monocytes/ macrophages, T lymphocytes, mast cells), of the arterial wall. This process leads to the development of complex lesions or plaques that protrude into the arterial lumen (4). It has been shown that endothelial cells upon activation, express adhesion molecules, that favor the attachment of monocytes which migrate into the

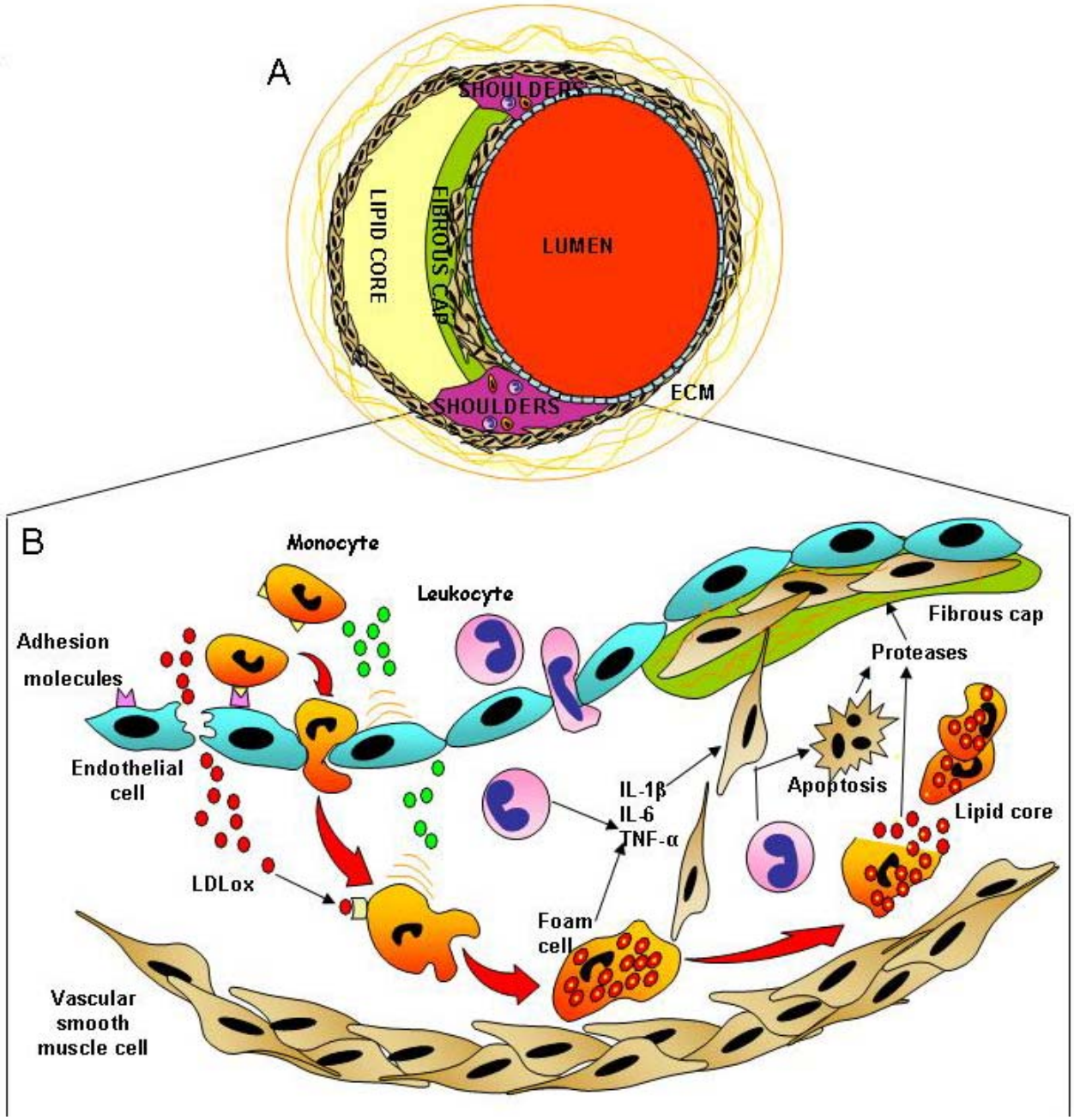

Figure 1. Atherothrombosis, a multifactor disease. A. Schematic representation of the different regions of human atherosclerotic plaques. B. The initial event in the atherosclerotic process is endothelial injury and the formation of fatty streaks originated by trapping of lipoproteins (LDL) and the appearance of leukocyte adhesion molecules on the endothelial cells, triggering monocyte infiltration. Monocytes migrate into the subendothelial space and differentiate into macrophages. Uptake of LDL (modified as oxidized LDL) via scavenger receptors leads to foam cell formation. Interactions between macrophage foam cells and T-lymphocytes establish a chronic inflammatory process, with the secretion of cytokines (such as TNF-α) and other pro-inflammatory mediators which attract SMCs and promote their proliferation. These cells synthesize extracellular matrix proteins that lead to the development of the fibrous cap. Necrosis or apoptosis of macrophages and SMCs result in the formation of a necrotic core and accumulation of extracellular cholesterol.

subendothelial space and differentiate into macrophages (5). Uptake of oxidized low density lipoprotein (oxLDL) by these macrophages, via scavenger receptors, leads to formation of foam cells (6). Interactions between macrophages foam cells and T-lymphocytes establish a chronic inflammatory process. Cytokines such as interleukin-1beta (IL-1β), interleukin-6

(IL-6), and tumor necrosis factor alpha (TNF-α) secreted by lymphocytes and macrophages, attract SMCs which proliferate and secrete extracellular matrix proteins, forming the fibrous cap.

In the more advanced phases, several mechanisms have been proposed to participate in the destabilization of atherosclerotic plaques. Macrophage and SMC cell death by apoptosis or necrosis leads to the formation of a necrotic core and accumulation of extracellular cholesterol (7). Moreover, the activation of different proteases contribute to the weakening of the fibrous cap (8,9). In addition, recent data have highlighted the role of intraplaque hemorrhage in the progression of human atheromatous disease (10-12). Furthermore, neovascularization has been identified as another potential process involved in the necrotic core expansion and intraplaque hemorrhage (13). All these mechanisms are involved in the destabilization and rupture of atherosclerotic plaques, which in turn exposes blood components to tissue factor, initiating coagulation, the recruitment of platelets and the formation of a thrombus. In the most complicated cases, the occlusion of an artery by the thrombus causes myocardial infarction, stroke or peripheral vascular disease.

3. TRAIL AND ITS RECEPTORS

The TNF-superfamily member APO2 ligand (APO2L) or TNF-related apoptosis-inducing ligand (TRAIL) was identified in 1995 by searching the human genome database for sequences with homology to TNF (14,15). Similar to other membrane-bound ligands of the TNF superfamily, TRAIL is a type II membrane protein which can be released in a vesicle-associated form (16) or in a soluble form that is generated through cysteine proteases shedding of the protein's extracellular, carboxy-terminal portion (17). However, unlike other ligands of the TNF-superfamily which expression is tightly regulated and temporally limited to activated cells, TRAIL mRNA has been detected in various cells and tissues (18).

Several receptors for TRAIL have been identified: two death receptors containing a death domain (DR4 and DR5, also called TRAIL-R1 and TRAIL-R2), and two decoy receptors (DcR1 and DcR2, also called TRAIL-R3 and TRAIL-R4) (19-25) (Figure 2). Like most of the known TNF superfamily receptors, TRAIL-R1, TRAIL-R2 and TRAIL-R4, are type I transmembrane proteins, whilst TRAIL-R3 is a type III transmembrane protein, lacking a signal peptide. Although TRAIL-R4 has a transmembrane domain, it contains a truncated death domain which renders it unable to efficiently transduce intracellular signals. A soluble receptor, osteoprotegerin (OPG), which was identified initially as a regulator of osteoclastogenesis that binds the receptor activator of nuclear-factor kB ligand (RANKL), was shown later to bind TRAIL (26).

The TNF-superfamily members have been widely studied in cancer due to their participation in the homeostatic regulation of the immune response and their potential use as cancer therapeutic agents (27). In this respect, TNF is involved in the protection of the organisms against infections, mainly promoting inflammatory responses of the cells. Other members of this family, such as TRAIL and Fas ligand (FasL), mediate apoptosis induced by natural killer cells and cytotoxic lymphocytes against different pathogens and tumor cells (28). In this regard, an increased susceptibility to tumor initiation and metastasis has been observed in TRAIL deficient mice (29). In contrast, most normal human cell types are resistant to TRAIL-induced apoptosis, making this ligand an attractive cancer therapeutic agent (30). However, TRAIL can also induce apoptosis of activated human T cells (31), neutrophils (32), macrophages (33), dendritic cells (34) and hepatocytes (35). Interestingly, this apoptotic response can be modulated by the binding of TRAIL to their receptors. In this respect, TRAIL-R3 has been proposed to act as an antagonizing decoy receptor and TRAIL-R4 was believed to negatively regulate TRAIL-induced cytotoxicity by competing for ligand binding with TRAIL-R1 and TRAIL-R2. However, a recent study (36) showed that inhibition of TRAIL-induced apoptosis by TRAIL-R4 depends on its association with TRAIL-R2 via the preligand assembly of both receptors, suggesting that TRAIL-R4 acts as regulatory receptor which inhibits apoptosis signalling by TRAIL through this novel ligand-independent mechanism. Finally, OPG has been shown to counteract the biological activities of TRAIL, since it promotes the survival of cancer cells by binding this ligand (37-41).

4. POTENTIAL IMPLICATION OF TRAIL IN THE MECHANISMS INVOLVED IN ATHEROSCLEROSIS

4.1. TNF superfamily and atherosclerosis

The majority of the members of the TNF superfamily has been detected in human atherosclerotic lesions (42-45), suggesting the potential implication of these proteins in atherothrombosis. TNF-α is a well-known mediator of systemic and vascular wall inflammation in atherothrombosis (46). Furthermore, TNF-α promotes human macrophage–induced vascular SMC apoptosis by cooperative interactions with nitric oxide (NO) and Fas/FasL (47). The Fas/FasL system plays a central role in atherothrombosis (43,48,49). In this regard, Fas expression has been detected in vascular SMCs of human atherosclerotic plaques and colocalized with apoptotic cells, implicating the Fas/FasL system in the control of viability of the plaque cells. In addition, FasL could be involved in the recruitment of inflammatory cells to the lesion, and overexpression of FasL in arteries of hypercholesterolemic rabbits can accelerate atherosclerotic lesion formation (50). However, Sata and Walsh (51) demonstrated that FasL expression by endothelial cells decreases inflammation in atherosclerotic lesions. Finally, therapeutic modulation of the different members of the TNF superfamily has demonstrated beneficial effects on different pathological processes associated to vascular injury, further supporting a main role of this family of proteins in the mechanisms underlying atherothrombosis (52,53).

4.2. TRAIL and atherothrombosis

TRAIL and its receptors are present in human atherosclerotic lesions (Figure 3). In this respect, TRAIL

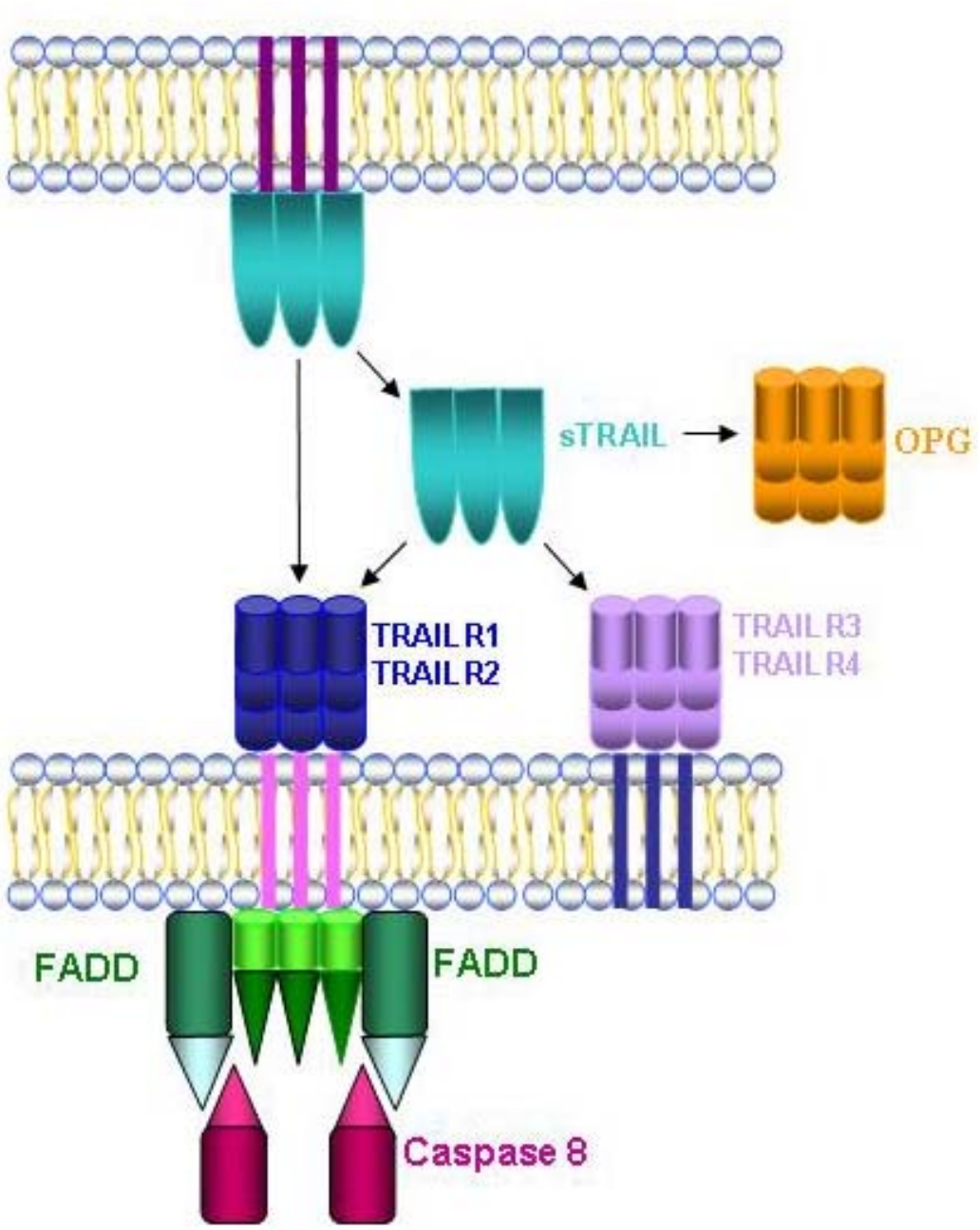

Figure 2. TRAIL and TRAIL receptors. TRAIL is a type II membrane protein, while TRAIL-R1, TRAIL-R2 and TRAIL-R4, are type I transmembrane proteins. TRAIL-R3 is a type III transmembrane protein, lacking a signal peptide. Although TRAIL-R4 has a transmembrane domain, it contains a truncated death domain which renders it unable to efficiently transduce intracellular signals. A soluble receptor, osteoprotegerin (OPG) has also been identified. FADD= Fas-associated death domain.

expression is increased in vulnerable plaques when compared to stable plaques and/or healthy mammary arteries (54). Interestingly, TRAIL-positive cells were mainly localized in the interface regions between the lipid core and the media and within the shoulder regions of the plaques, where inflammatory cells are predominantly known to reside. In relation to TRAIL receptors, TRAIL-R2 has been detected in vascular SMCs of human atherosclerotic plaques (55). Interestingly, TRAIL-R2 was expressed on vascular SMC in the cap region, but not on vascular SMC in normal carotid artery walls. In addition, OPG was observed in the nondiseased vessel wall and in early atherosclerotic lesions (56). Furthermore, it has been shown that OPG is expressed at high levels in symptomatic human carotid atherosclerotic plaques (57). More recently, strong immunostaining of OPG was seen within thrombus material obtained at the site of plaque rupture during acute myocardial infarction (58).

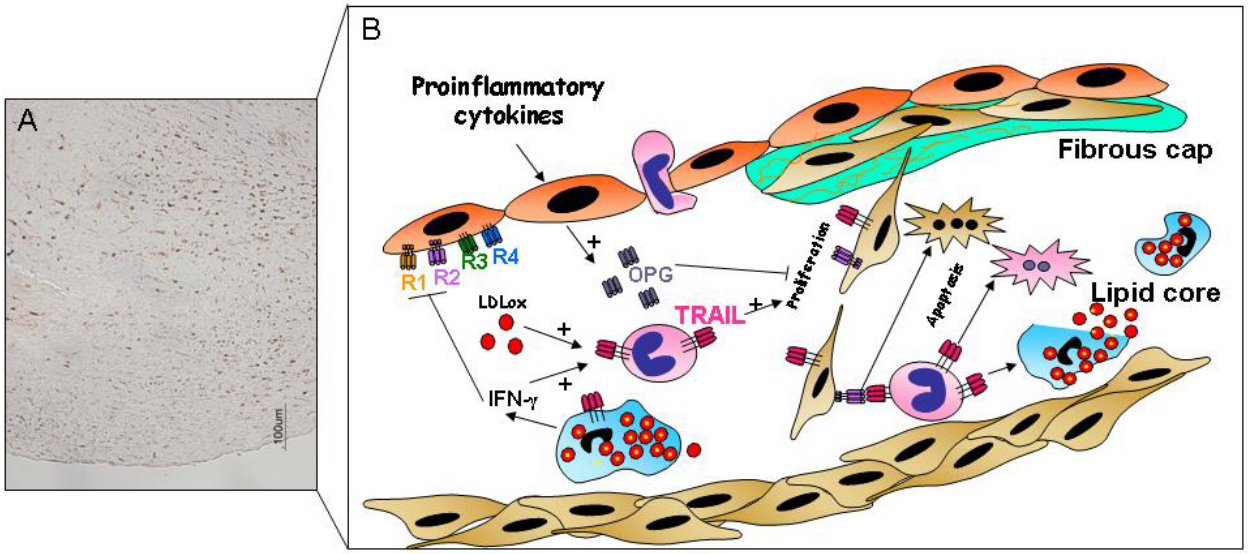

Figure 3. TRAIL and atherothrombosis. A. Representative photograph of TRAIL immunodetection in human carotid atherosclerotic plaques.B. Schematic representation of the potential stimuli present in atherosclerotic plaques modulating TRAIL and TRAIL receptors (R1-R4) expression. The potential proatherogenic actions induced by the interaction between TRAIL and TRAIL receptors in human atherosclerotic plaques is also shown. IFN-γ=interferon gamma, oxLDL= oxidized low density lipoprotein.

TRAIL expression can be induced by different stimuli present in the atheroma plaque, such as IFN-γ (59) or oxLDL (54). In contrast, IFN-γ significantly suppressed the expression of TRAIL-R1 and TRAIL-R2 on human endothelial cells (60). Finally, OPG expression is markedly upregulated in response to inflammatory cytokines in endothelial cells (61).

4.2.1. Proliferation

The initial steps of atherosclerosis are characterized by the proliferation and migration of vascular SMCs from the media towards the intima. In this respect, TRAIL induces the proliferation and migration of vascular SMCs (62). Of note, all the biological effects induced by TRAIL were significantly inhibited by pharmacological inhibitors of the ERK pathway. In contrast, incubation of human vascular SMCs with increasing concentrations of recombinant OPG induced a dose-dependent reduction in DNA synthesis (63). The inhibition of vascular SMC proliferation by recombinant OPG coincided with an additional effect of the cytokine in suppressing vascular SMC production of the SMC mitogen IL-6.

On the whole, these results could suggest the potential contribution of TRAIL in the neointimal formation of atherosclerotic plaques; however, there is not yet *in vivo* data which could support this hypothesis.

4.2.2. Inflammation

Inflammation is involved in the initiation, progression and destabilization of atherosclerotic plaques. The breakdown of the plaque occurs more frequently at points where the fibrous cap is thinner and where there is a great amount of inflammatory cells such as macrophages and T lymphocytes (64,65).

The activation of the endothelial cells appears to play a pivotal role in the initiation of the inflammation process. In response to tissue injury, endothelial cells express adhesion molecules that mediate the binding of activated leucocytes to the endothelial surface and their extravasation. In this respect, while some authors have shown that TRAIL may induce inflammatory gene expression on endothelial cells (66), other suggests that TRAIL counteract the adhesion of monocytes to endothelial cells by downregulating the expression of the inflammatory chemokines CCL8/MCP-2 and CXCL10/IP-10 (67). In addition, Zauli et al observed that TRAIL stimulation of endothelial cells did not affect the expression of the proinflammatory enzyme cyclooxygenase-2, but upregulated the release of Prostaglandin E_2 (68), which have been implicated in the instability of atherosclerotic plaques (69,70).

The expression of some of the molecules mentioned above is transcriptionally regulated by nuclear factor-kB (NF-kB). NF-kB is a key mediator involved in the early and late stages of plaque instability and rupture (71). It is important to note that increased NF-kB activation has been observed in the vulnerable region of the shoulders of the plaques (49), where TRAIL has been mainly localized (54). In some cells, TRAIL binding to TRAIL-R1 and R2 activates NF-kB (66,72). Upon TRAIL binding, both TRAIL receptors bind the adaptor molecule Fas-associated death domain (FADD), which may explain the potent activation of NF-kB observed by TRAIL receptors. On the contrary, other authors have demonstrated that TRAIL has no effect on NF-kB activation (68,73,74).

On the whole, it is not clear the potential global effect of TRAIL on inflammation *in vivo*. In this respect,

the inyection of TRAIL to human skin xenografts on mice, promoted vascular injury and leukocyte infiltration (66). However, to the best of our knowledge, recombinant TRAIL has not yet been used in animal models of atherosclerosis.

4.2.3. Neovascularization

Neovascularization consist in the formation of new blood vessels mediated by progenitor and/or endothelial cells. Angiogenesis, the predominant form of neovascularization in atherosclerosis, is mediated by endothelial cells coming from venules and leads to the formation of new capillaries (75). In this respect, neovessels can be involved in the recruitment of leukocytes to vulnerable areas of the plaques, including the cap and shoulders (76). Moreover, ruptured plaques exhibited the highest degree of neovascularization (77).

In this respect, it was observed that TRAIL induces the migration of endothelial cells, differentiation into tube like structures in matrigel assays and release of nitric oxide (NO), similar to vascular endothelial growth factor (VEGF), a well known angiogenic factor (74). These results differ from those recently published, where TRAIL showed antimitogenic effects on human umbilical vein endothelial cells (HUVECs), inhibiting new vessel formation in the in vitro matrigel model and exerting a powerful inhibition of blood vessel formation induced by an angiogenic cocktail administered *in vivo* in mice. (78). In addition, it has been demonstrated the ability of OPG to induce the formation of cord-like structures in vitro using a matrigel tubule formation assay (79).

Degradation of matrix by activated matrix metalloproteinases (MMPs), detectable in vessels undergoing remodelling, is thought to facilitate cell migration and general reorganization of vascular tissue. There is not information regarding the potential effect of TRAIL on MMP expression in vascular cells. However, in a recent study, TRAIL inhibited mRNA expression of MMP-2 in different human glioblastoma cell lines (78). In contrast, it has been observed that recombinant OPG induced MMP-9 expression in vascular SMCs and monocytes (63).

4.2.4. Apoptosis

The most studied role of TRAIL, mainly by the interaction with its receptors TRAIL-R1 and TRAIL-R2, is the induction of apoptosis. TRAIL binds to type I transmembrane proteins such as TRAIL-R1 and TRAIL-R2 and triggers the formation of the death-inducing signalling complex that recruits procaspase-8 through the adaptor protein Fas-associated death domain (FADD) (Figure 2). Proteolytically activated caspase-8 mediates the activation of terminal effector caspase-3 and causes target cell death.

Apoptosis of vascular cells plays a central role in atherogenesis. In the early stages of atherosclerosis, loss of endothelial cells can increase the permeability of the vessel wall to lipids and inflammatory cells and induce the proliferation of vascular SMCs; in contrast, loss of vascular SMCs and macrophages could have some beneficial effect in preventing plaque progression. In the more advanced stages, diminution of vascular SMCs from the cap of atherosclerotic plaques could have deleterious consequences, since vascular SMCs are the main source of matrix proteins, which are determinant for plaque stability. Specimens from unstable coronary plaques obtained by atherectomy show an increase in apoptotic vascular SMCs and a decrease in the total number of these cells (80). Furthermore, extensive macrophage apoptosis has been shown at the site of plaque rupture in autopsy specimens from people who suffered sudden coronary death (81). In early lesions, the absolute number of apoptotic cells could be underestimated given that apoptotic cells are rapidly cleared by macrophages *in vivo*. In late lesions, however, different factors may contribute to defective phagocytic clearance of apoptotic macrophages, leading to secondary necrosis of these cells and a proinflammatory response (82). Thus, not only apoptosis itself but also the defective functionality of lesional phagocytes to safely clear apoptotic cells may be important determinants of plaque instability and rupture.

There is controversial data in relation to the potencial role of TRAIL in endothelial cell apoptosis. While some authors have shown that TRAIL may induce apoptosis of endothelial cells (60, 66,83), others reported no apoptosis (73) or even survival of endothelial cells (74). In this regard, particular conditions seem to be necessary to allow TRAIL to induce apoptosis in endothelial cells, such as the inhibition of PI3 kinase prosurvival pathway (74). Among the mechanisms involved in protection of cells against TRAIL induced apoptosis, several options have been proposed: increased expression of TRAIL decoy receptors, low surface death receptor expression and inhibitory mechanisms downstream of activated caspase-3. A key point involved in the proapoptotic effect of TRAIL is the modulation of the expression of its receptors. In this respect, another study has shown that IFN-γ pretreatment significantly suppressed the expression of TRAIL-R1 and TRAIL-R2 on HUVECs, and inhibited apoptosis in response to TRAIL (60).

In addition, TRAIL has been shown to induce the death by apoptosis of other cells present in the atherosclerotic plaque, such as lymphocytes, macrophages and vascular SMCs (31,33,55). In a recent study, it has been shown that patients with acute coronary syndromes (ACS) bear increased frequencies of CD4 T cells that express TRAIL upon stimulation and that adoptive transfer of such CD4 T cells causes vascular SMC death in human atherosclerotic plaques, demonstrating the *in vivo* relevance of this mechanism in plaque destabilization (55). In this study, CD4 T cells equipped to induce apoptosis of cultured vascular SMCs not only accumulated in the plaque, but also circulated in the blood of patients with ACS. Finally, *in vivo* studies confirmed the ability of CD4 T cells to infiltrate into the lesion and mediate vascular SMC killing.

On the whole, these results suggest that TRAIL can contribute to the instability of atherosclerotic plaques

by the induction of apoptosis of resident and inflammatory cells, a main determinant of plaque stability.

5. BIOMARKERS OF CARDIOVASCULAR DISEASES

Being able to predict who is at risk of an acute thrombotic event is at present one of the major challenges of cardiovascular medicine. Although it is well-known that classical risk factors (abnormal lipid profile, diabetes, smoking or hypertension) or a previous history of clinical atherothrombosis, confer a high probability of future events, additive prognostic markers are needed to predict more accurately the global cardiovascular risk. While noninvasive imaging techniques are being investigated to improve characterization of the size and morphology of the atherosclerotic plaques, the other field of growing interest is the search of potential prognostic biomarkers which can be identified in blood (84,85). The most extensively studied potential biomarker to date has been C-reactive protein (CRP) (86). Although it could be of use in some risk situations, it appears to have only a moderate predictive value (87), and it has not gained wide acceptance in clinical practice. In addition to CRP, many different proteins, mainly involved in inflammation, have been studied in recent years as potential candidates for risk prediction. Among these are CD40L, monocyte chemoattractant protein-1 (MCP-1), adhesion molecules, myeloperoxidase, and several interleukins. Nevertheless, none of them has been consistently demonstrated to add predictive value to the clinical variables used in the clinical practice and, in most cases, there are no commercially available standardized assays (88).

As previously mentioned, the extracellular region of many of the TNF-superfamily ligands is proteolytically processed into a soluble form of the protein that is released to the extracellular space. Plasma concentrations of TNF-α are directly associated with the degree of early carotid atherosclerosis (89). We previously observed that soluble FasL levels are decreased in patients with combined hyperlipemia and carotid atherosclerosis (90). In addition, serum levels of soluble TRAIL were reduced in patients with ACS compared to patients with stable angina pectoris and/or healthy subjects (54). Moreover, these authors observed a negative correlation between TRAIL and CRP levels. In a more recent study, TRAIL serum levels tended to be lower in patients with coronary artery disease (CAD) compared to those without CAD (91). Since OPG has been shown to counteract the biological activities of TRAIL, an elevation of circulating OPG levels may represent a crucial compensatory mechanism to limit the effects of TRAIL (Figure 4). In this respect, elevated OPG serum levels have been reported in patients with CAD (92), unstable angina (58), carotid atherosclerosis (93) and heart failure (94). Moreover, OPG serum levels positively correlated with the severity of CAD or peripheral artery disease (92,93,95). Finally, elevated OPG serum levels are associated with increased cardiovascular mortality (96). Interestingly, circulating OPG levels have prognostic value in patients with peripheral artery disease, abdominal aortic aneurysm and heart failure (63,97).

Special attention has been directed recently towards the potential use of OPG as a surrogate marker to identify diabetic patients. It is important to remember that diabetic patients are treated as high risk patients since the presence of diabetes per se increase three fold the risk of CAD. In this respect, elevated OPG serum levels are associated with glycaemic status, systolic blood pressure, kidney function and cardiovascular morbidity in type 1 diabetic patients (98). Similarly, in type 2 diabetic patients, OPG serum levels have been related to vascular endothelial dysfunction (99) and with silent CAD (100).

6. CONCLUSIONS

Interaction between members of the TNF superfamily and their receptors is mainly involved in the homeostatic regulation of the immune response. However, this family of proteins could mediate other mechanisms underlying atherosclerotic plaque development, such as proliferation, inflammation, neovascularization and apoptosis. In this respect, there are still some controversial data on the effects of TRAIL on inflammation and apoptosis of vascular cells. The rationale for the differences observed in these experiments could result from the cells studied (cell lines or primary cultures), the methods used to asses these processes and the composition, potency and toxicity of the recombinant preparations of TRAIL. However, recent *in vivo* studies have suggested a potential proinflammatory and proapoptotic role of TRAIL in vascular injury.

TRAIL and some of its receptors (TRAIL-R2 and OPG) have been detected in human atherosclerotic plaques. TRAIL could mediate its biological effects inside the atherosclerotic plaques trough TRAIL-R2. In contrast, TRAIL-R2 has not been observed in healthy arteries, which could suggest the potential modulation of the effects of TRAIL through the differential expression of its receptors in pathological vs healthy arteries. However, there is no information about the presence of other TRAIL receptors in human atherosclerotic plaques or healthy arteries, except for OPG. In this respect, it is interesting to note that *in vitro* studies have demonstrated opposing effects of TRAIL and OPG in the different mechanisms involved in atherosclerosis, which could suggest another potential mechanism to modulate the effects of TRAIL in human arteries.

Finally, the evaluation of TRAIL and OPG levels as potential cardiovascular biomarkers has gained recent attention. In this respect, while TRAIL levels are decreased in patients with CAD, elevated OPG levels have been reported in patients with different cardiovascular diseases. In this respect, since the relative concentrations of TRAIL and OPG are key determinant in TRAIL/OPG interactions, an elevation of circulating OPG levels may represent a crucial compensatory mechanism to limit the effects of TRAIL.

Future studies in animal models of atherosclerosis are needed to asses the final contribution of TRAIL in the pathogenesis of this disease. In addition, the modulation of

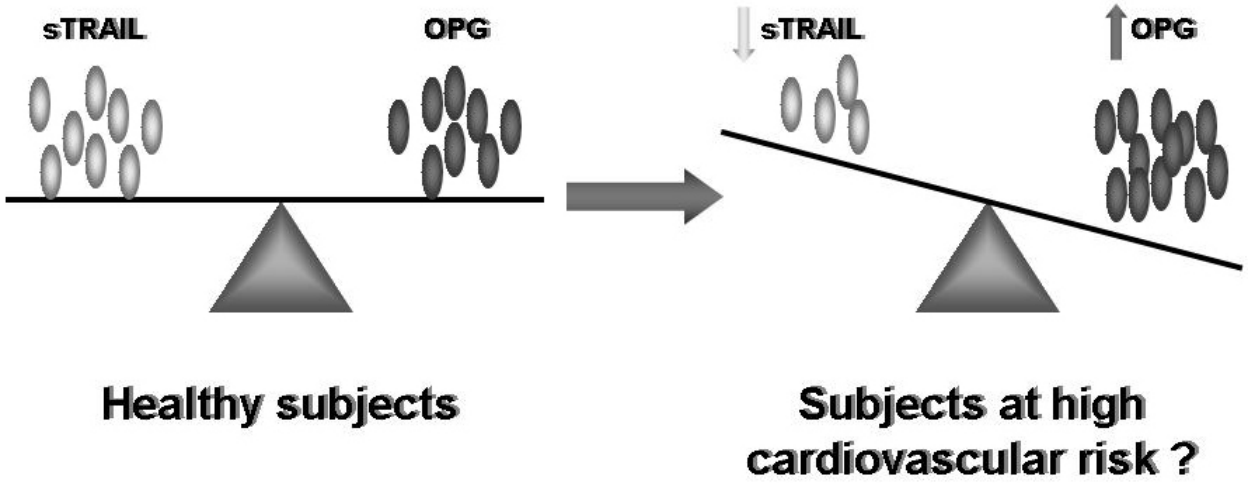

Figure 4. TRAIL and OPG plasma levels in cardiovascular diseases. While soluble TRAIL (sTRAIL) levels are decreased in the plasma of patients with CAD, elevated OPG levels have been reported in patients with different cardiovascular diseases. It could be hypothesized that an elevation of circulating OPG levels may represent a crucial compensatory mechanism to limit the effects of TRAIL.

the interaction of TRAIL with its receptors *in vivo* could help us to understand the role of this system on vascular injury. Finally, the potential use of TRAIL/OPG as prognostic/diagnostic markers of cardiovascular diseases must be further evaluated in large population studies.

7. ACKNOWLEDGEMENTS

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