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TWEAK and Fn14. New players in the pathogenesis of atherosclerosis

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1. ABSTRACT

Atherosclerosis is currently described as an inflammatory disease given that the main components of chronic inflammation are present in this process: cell recruitment, proliferation, neovascularization, and sclerosis. Vascular lesions are caused by inflammatory and fibroproliferative responses to injury of the endothelium and vascular smooth muscle cells. Interaction between members of the tumor necrosis factor (TNF) superfamily and their receptors elicits diverse biologic actions that participate in atherosclerosis development. These responses include the expression of adhesion molecules, proinflammatory cytokines, matrix metalloproteinases, and tissue factor, which are known to increase plaque instability. TNF-like weak inducer of apoptosis (TWEAK) is a recently described member of the TNF superfamiliy, which is involved in induction of inflammation, activation of cell growth, and stimulation of apoptosis. In this review, we summarize the potential proatherogenic consequences of the interaction of TWEAK with its receptor Fn14 in the vascular wall.

2. INTRODUCTION

Atherosclerosis and its complications still represent the major cause of death in developed countries. It has been appreciated for decades that this disease is linked to hypercholesterolemia and the accumulation of inflammatory cells in the artery wall, although the exact mechanisms underlying this process remain unclear. Atheroclerotic lesions begin as fatty streaks in the subendothelial space of large arteries. Recruitment of monocytes/macrophages and their subsequent uptake of modified low density lipoprotein (LDL) - cholesterol contribute to fatty streak formation. The recruitment of monocytes to lesion-prone sites is regulated by cell adhesion molecules that are expressed on the surface of endothelial cells in response to inflammatory stimuli. Interactions between macrophages and T lymphocytes lead to a chronic inflammatory response. Cytokines and chemokines secreted by these cells induce proliferation and migration of vascular smooth muscle cells (SMCs) from the media and subsequent neointima formation (1).

In a more advanced stage, vulnerable plaques are characterized by a fibrino-lipido-cruoric-core, encapsulated between the cap and the remaining media. This cap confers to the lesions resistance to rupture, and consists of collagen and other extracellular matrix (ECM) proteins, synthesized by vascular cells. Expression of different proteases leads to degradation of ECM proteins and promotes plaque instability. Furthermore, repeated intraplaque hemorrhages conveying leukocyte proteases and plasma zymogens foster the progression of the core toward rupture. Rupture of an atherosclerotic plaque may result in the occlusion of an artery by the formation of a thrombus over an atherosclerotic lesion, causing myocardial infarction, stroke or peripheral vascular disease (2).

3. TWEAK AND Fn14

3.1. Structure

The cytokine tumor necrosis factor-like weak inducer of apoptosis (TWEAK, Apo3L, TNFSF12) is a member of the tumor necrosis factor superfamily (TNFSF) described in 1997 (3). The human TWEAK gene is located at chromosomal position 17p13.1 and encodes a 249-amino acid protein (3). TWEAK is a type II transmembrane protein, with the N-terminal region containing a hydrophobic anchor which allows its insertion into the cell membrane with the N-terminus inside the cell. This cytoplasmatic portion of TWEAK contains a putative serine phosporylation site. The C-terminal extracellular domain contains the receptor-binding site and, as other members of the TNF superfamily, has a consensus sequence motif for furin cleavage. TWEAK is initially synthesized as a membrane-bound protein that is processed quickly into a smaller, soluble fragment which mediates the different biological properties of this molecule.

TWEAK receptor was initially reported to be the TNFSF member named DR3 (4). However, different subsequent studies failed to confirm a TWEAK-DR3 interaction. Moreover, other reports have demonstrated that TWEAK can bind to different cells that do not express DR3 (5-6), indicating that other protein/s were the receptor/s of TWEAK. Using an expression cloning strategy employing a cDNA library of human endothelial cells, Wiley *et al.* identified a novel receptor for TWEAK that was named TWEAK receptor (TweakR). The DNA sequence analysis demonstrated that TweakR was identical to human fibroblast growth factor-inducible 14 (Fn14) (7), and different experiments confirmed that TWEAK binds to Fn14 (8). The human Fn14 gene is located at the chromosomal position 16p13.3 (9) and encodes a 129-amino acid protein. Fn14 is a type I transmembrane protein that is synthetized with a N-terminal signal peptide which is processed into a mature form of the protein. More details of the Fn14 and TWEAK structure are available in the review of Wiley *et al.* (8).

Human and murine TWEAK are closely related, with a 93% homology in the receptor-binding domain. Furthermore, human and murine Fn14 have a 90% homology in their overall sequences. This detail is of importance since it has recently been published that TWEAK does not cross-react with any other members of the TNF or TNFR superfamilies, its interaction being specific for Fn14. In addition, human TWEAK can bind to murine Fn14 and vice-versa (10).

The signalling induced by TNF superfamily receptors usually involves the presence of death domains in their cytoplasmatic tail. However, Fn14 cytoplasmatic tail is too short to have a death domain but contains a TRAF-binding site with three threonines that could be potentially phosphorylated and induce TRAF binding and subsequent transmission of TWEAK signalling (11).

Although Fn14 is the only identified TWEAK receptor, Polek *et al.* have recently proposed that some biological effects of TWEAK may be mediated independently of its interaction with Fn14 in RAW264.7 cells (monocytes/macrophages) (12), indicating the possibility that additional receptors are implicated in the transduction of TWEAK signalling. However, this report has not been confirmed.

3.2. TWEAK and Fn14 expression

TWEAK and Fn14 expression have been documented in several tissues and cell lines. TWEAK mRNA is expressed in pancreas, intestine, heart, brain, lung, ovary and skeletal muscle, and at lower levels in liver and kidney (3, 13). High levels of Fn14 mRNA have been detected in kidney, heart, lung and placenta (7-9). TWEAK mRNA has been detected in different cultured cells such as human peripheral blood lymphocytes, mouse peritoneal macrophages, human endothelial cells, mesangial cells and SMCs. In addition, Fn14 mRNA is expressed by human endothelial cells, SMCs, fibroblasts, monocyte/macrophages, astrocytes and mesangial cells in culture. However, it is important to note that TWEAK or Fn14 mRNA expression levels may not be related to protein levels. In this sense, although TWEAK mRNA is detected in lymphocytes, the cytokine is not found on the membrane of these cells. Furthermore, although SMCs express Fn14 at the mRNA level (14), the protein has not been detected in quiescent SMCs in culture (15). As far as the vascular wall is concerned, TWEAK is expressed in normal non-atherosclerotic arteries whereas Fn14 expression is almost absent (Figure 1). Moreover, Fn14 and TWEAK are expressed in different areas of atherosclerotic plaques and these proteins colocalize with SMCs and macrophages (Figure 2). Furthermore, Fn14 and TWEAK colocalize within the same cell (15) indicating that Fn14/TWEAK interaction could be present in the diseased vessel wall, which may have adverse outcomes in atherosclerotic lesions.

4. POTENTIAL PROATHEROGENIC ACTIONS OF TWEAK/Fn14

The interaction of TWEAK with Fn14 elicits multiple biological responses including stimulation of proliferation, migration, apoptosis, angiogenesis, and induction of proinflammatory cytokines. All these processes participate in the different steps of atherosclerotic plaque development. We now summarize the potential role of TWEAK/Fn14 in all phases of the atherosclerotic process: initiation, progression, plaque destabilization and subsequent thrombosis.

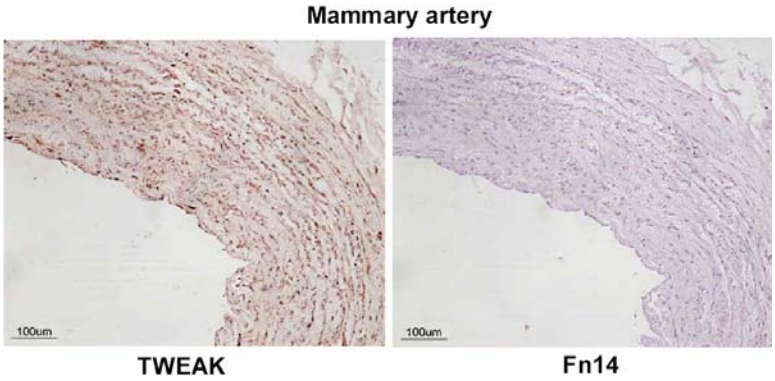

Figure 1. Expression of TWEAK and Fn14 in human non-atherosclerotic mammary arteries. Representative immunohystochemistry microphotography. Original magnification x100.

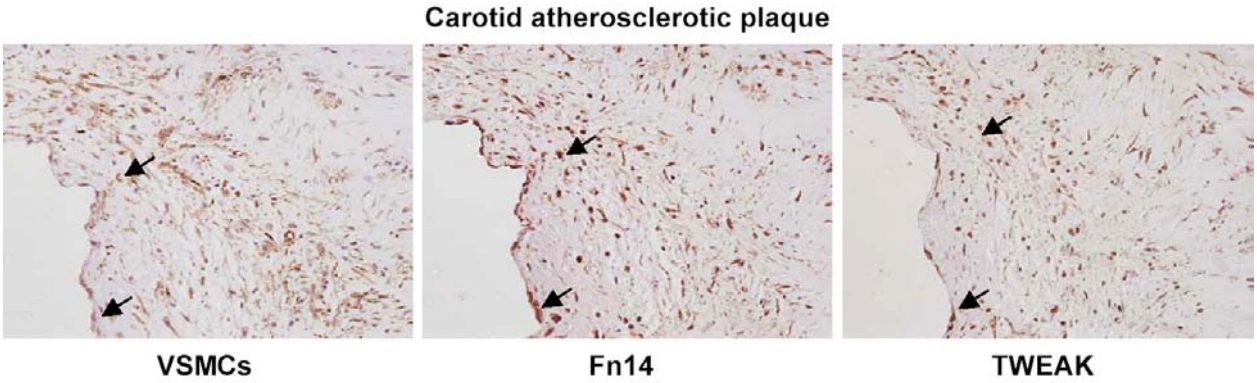

Figure 2. Colocalization of TWEAK, Fn14 and vascular smooth muscle cells. Immunohystochemistry showing TWEAK, Fn14 and alpha-smooth muscle actin expression (arrows) in serial sections of human carotid atherosclerotic plaques. Original magnification x 200.

4.1. Initiation of atherosclerotic plaque formation: Endothelial dysfunction

Healthy endothelium does not bind circulating cells probably, in part, by action of the Fas/Fas ligand system (16,17), another members of the TNFR/TNF superfamilies. Fas ligand is constitutively expressed on normal ECs and may exert an atheroprotective function through its ability to induce apoptosis in mononuclear cells attempting to invade the vessel wall in the absence of normal inflammatory stimuli. However, under pathological conditions, endothelial cells acquire an activated phenotype by the expression on their surface of adhesion molecules such as intercellular adhesion molecules (ICAMs), selectins, and vascular adhesion molecules (VCAMs) that act as receptors for proteins expressed by leukocytes (monocytes and lymphocytes, neutrophils), and the diminution of FasL expression. In this sense, one of the early stages in atherogenesis is the adhesion of monocytes to the endothelium. This activation is believed to be related to a decrease in nitric oxide (NO) availability, which may be secondary to different cardiovascular risk factors (hypertension, dyslipidemia, diabetes, and so forth) (18). In particular, expression of VCAM-1, ICAM-1 and selectins participate in the recruitment of monocytes and T lymphocytes that can be found in the early human and animal experimental lesions. After that, inflammatory cells begin their migration into the arterial wall, with the participation of different chemoattractant proteins, such as monocyte chemoattractant protein-1 (MCP-1). This chemokine is responsible for the migration of monocytes into the intima where they are converted into macrophages, which express scavenger receptors for modified lipoproteins (19). Ingestion of lipoproteins by macrophages induces the differentiation of these cells into foam cells and causes the formation of a fatty-streaks, the first lesion of atherosclerosis.

TWEAK/Fn14 could participate in the initiation of atherosclerotic lesions through the induction of the expression of adhesion proteins. Interaction of TWEAK with its receptor can induce ICAM-1 and E-selectin expression in human umbilical endothelial cells (HUVEC) in culture (20). In this sense, an antibody which blocks the TWEAK/Fn14 interaction totally inhibited the expression of the aforementioned adhesion molecules, indicating that Fn14, which is constitutively expressed, mediates TWEAK-induced expression of adhesion molecules in HUVEC. Furthermore, stimulation of HUVEC with TWEAK also induces the secretion of proinflammatory cytokines and chemokines such as interleukin-8 and MCP-1 that could also participate in the development of the atherosclerotic plaque by attracting neutrophils and monocytes.

4.2. Inflammatory response in atherosclerosis

Foam cells and lymphocytes present in fatty streaks secrete different cytokines that promote SMC proliferation and migration and the synthesis of a dense surrounding ECM, leading to intimal thickening. These SMCs change their phenotype and secrete factors that increase the recruitment of additional inflammatory cells, perpetuating the inflammatory response. The proliferation and migration of SMCs, recruitment of monocytes/macrophages and overproduction of ECM lead to intermediate lesions. Finally, the advanced atherosclerotic lesions are formed by a necrotic core composed of apoptotic and necrotic cells, characterized by its lipid moiety coming from trapped LDLs and foam cells which release their lipid content (2).

The role of TWEAK/Fn14 in the perpetuation of atherosclerotic plaques have been related to their ability to induce proliferation and migration of different vascular cells (21-22). Incubation of recombinant TWEAK with endothelial cells induces their proliferation and migration, effects mediated by Fn14 (20-21). Moreover, recombinant TWEAK also increases human aortic SMCs proliferation in culture (21). Furthermore, overexpression of the wild type Fn14 in SMCs increased migration of SMCs (23). In contrast, migration of cells expressing Fn14 mutants was inhibited relative to the levels of control cells. All these data suggest that TWEAK, through the interaction with its receptor, could be implicated in the intimal thickening observed during atherosclerotic plaque development.

4.2.1. Linking TWEAK to plaque inflammation

A variety of pathophysiological situations that affect cells of the vasculature, including endothelial and SMCs, leads to the expression of genes such as adhesion molecules and chemokines that are dependent on members of the nuclear factor (NF)-kappaB family of transcription factors. These proteins are redox-sensitive transcription factors that are involved in the transmission of various signals from the cytoplasm to the nucleus of numerous cell types (24). It is found as a trimer consisting of p50, p65 and IkB subunits in the cytosol. When NF-kB is activated, IkB is phosphorylated and released from the trimer, resulting in the migration of the p50/p65 heterodimer to the nucleus and the subsequent DNA binding (25). This process activates genes involved in the immune, inflammatory or acute phase response, such as cytokines (MCP-1, interleukin-8), adhesion molecules and procoagulant proteins [tissue factor (TF), plasminogen activator inhibitor 1 (PAI-1)]. A variety of stimuli have been found to induce NF-κB activation, including phorbol myristate acetate, ox-LDL and cytokines such as TNF-α, lymphotoxin and IL-1. Recent data strongly suggest that NF-kB could be involved in the pathogenesis of atherosclerosis (26). NF-kB is present in human atherosclerotic lesions in the nuclei of macrophages and endothelial cells (27), and participates in dysregulation of SMCs in human atherosclerosis (28). A characteristic of Fn14 is that it contains a binding domain for TRAFs, adaptor cytoplasmatic molecules that activate downstream signalling (11). Fn14 can bind to four different TRAF proteins, TRAF 1, 2, 3 and 5 (11, 23). This association activates different intracellular pathways, including NF-kB, JNK, ERK and p38 activation (8). The TWEAK/Fn14 interaction can activate NF-kB pathway in diverse cell types (11, 23, 29-32). This activation has been related with the phosphorylation of the inhibitory subunit IkB by the action of IkB kinase (IKK) (32), and its subsequent degradation (29), inducing the expression of different proinflammatory proteins including IL-6, IL-8, RANTES and ICAM-1, all of them implicated in the atherosclerotic plaque development. In addition, TWEAK also induces the release of MCP-1 from SMCs that could contribute to the increment of monocyte/macrophage infiltration into the vascular lesions (15) (Figure 3).

Whether other signal transduction cascades could be also activated by TWEAK/Fn14 interaction is unknown. At present, only one study has reported that using an inhibitor of p38 MAP kinase, the induction of IL-6 andIL-8 release induced by TWEAK in human astrocytes could be diminished (33). Interaction between TWEAK and its receptor also increases the phoshorylation of ERK 1/2 and JNK 1/2 (8). These data indicate that multiple signalling cascades are implicated in the biological effects of TWEAK in different cells types.

4.3. Plaque destabilization

The stability of the atherosclerotic plaque depends greatly on the integrity of the fibrous cap, which in turn depends on its ECM protein components. Plaque rupture occurs in areas of the atherosclerotic lesions with sustained inflammation, macrophage accumulation and apoptosis, mainly in the shoulders of the lesion. Advanced atherosclerotic lesions usually have a dense fibrous cap. However, SMCs and macrophages increase metalloproteinases (MMP) production, which are responsible for collagen degradation present in the fibrotic cap, rendering the atherosclerotic plaque susceptible to rupture. Activated T-cells may also contribute to plaque instability by stimulating MMP production by SMCs (34). Furthermore, these T lymphocytes produce interferon-γ (INF-γ) that can decrease collagen production by SMCs, leading to the weakening of the fibrous cap (35).

The expression of TWEAK and Fn14 has been detected in macrophage/foam cell rich regions of atherosclerotic plaques (36). The expression patterns of Fn14 and different MMPs overlapped in the same regions of atherosclerotic plaques (37). Kim *et al.* reported that incubation with an anti-TNFRSF12 antibody increased the expression of MMP-1/13 and MMP-9 (which degrade fibrillar and non-fibrillar collagen respectively) in activated monocytes. Furthermore, the addition of TWEAK also increased MMP-9 and MMP-2 protease activity in cultured monocytes (36). All these data indicate that the interaction of TWEAK with its receptor could participate in ECM degradation and favours plaque instability.

Another characteristic of the atherosclerotic plaque is the presence of apoptotic cells (26). Loss of cells inside the atherosclerotic plaque can also contribute to its instability. In this sense, interaction between TWEAK and Fn14 is responsible for cell death induction in different situations. TWEAK has proapoptotic activity on different tumor cell lines and on monocytes (6). TWEAK-induced

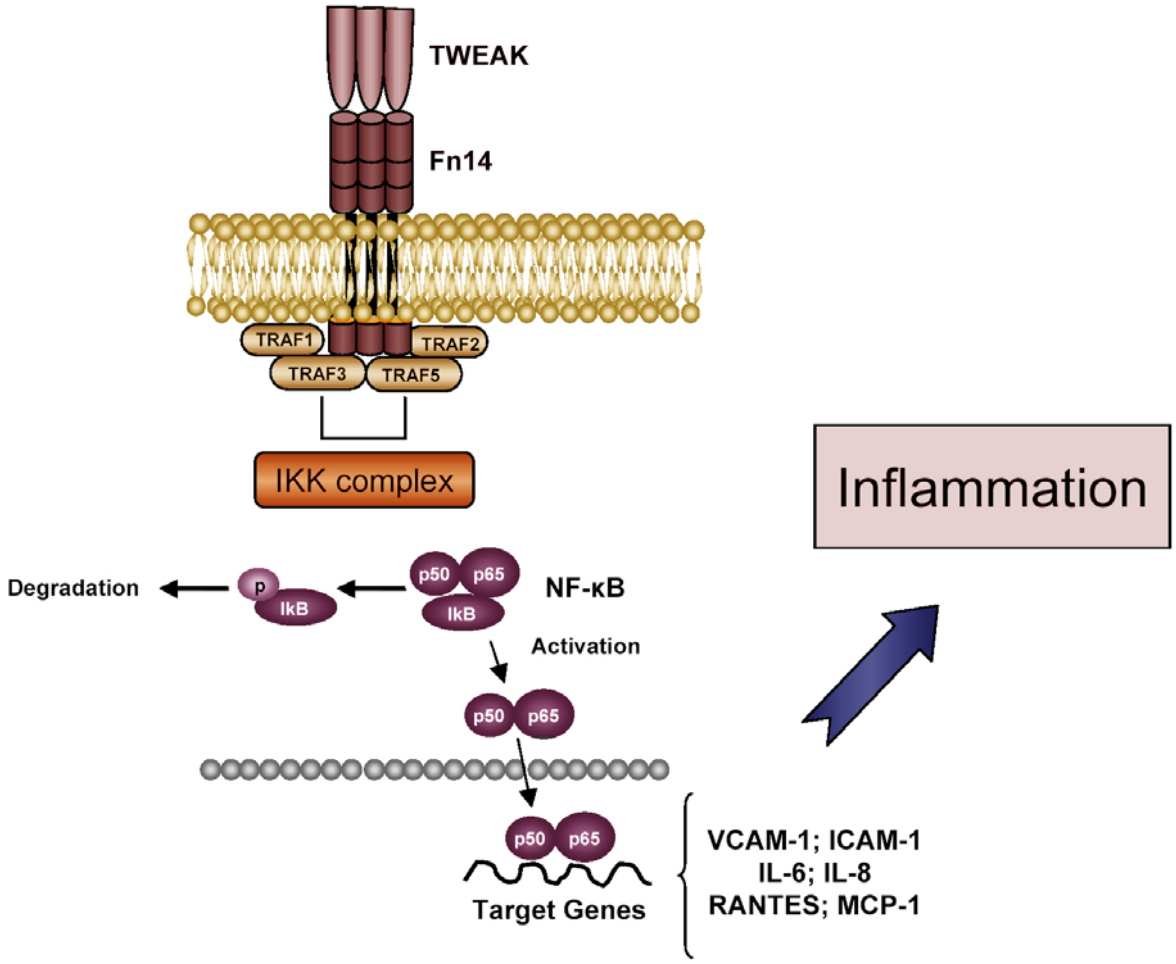

Figure 3. TWEAK induces a proinflammatory response. Schematic representation the potential inflammatory pathway induced by TWEAK. Interaction of TWEAK with Fn14 induces the recruitment of TRAF and activation of NF-kB that enhances the expression of different proteins that are implicated in inflammation.

apoptosis frequently requires the presence in the microenvironment of additional factors, such as INF-γ (38). TWEAK is not expressed in human peripheral blood mononuclear cells (PBMCs). However, upon INF-γ stimulation, high levels of TWEAK are detected in PBMCs. In this context, INF-γ-stimulated monocytes can induce apoptosis of target cells, indicating that TWEAK is involved in monocyte cytotoxicity (39). In this situation, it is possible that SMCs expressing Fn14 under proinflammatory conditions (15) could be a good target for TWEAK-induced cytotoxicity. However, this characteristic of TWEAK related with cells implicated in the atherosclerotic plaque development need to be investigated. The mechanisms by which TWEAK induces apoptosis remain undefined. Two different pathways could be involved in apoptosis induced by TWEAK. One of them is the activation of caspases such as caspase 8 and caspase 3 which are responsible for the cell death (6). However, other caspase-independent mechanisms have been reported, in which TWEAK induces cell death by action of cathepsin B (6, 40).

5. THERAPEUTIC MODULATION OF TWEAK/Fn14

The potential role of TWEAK/Fn14 in the development of atherosclerotic lesions should be confirmed in experimental models of atherosclerosis in which different therapeutic approaches to the modulation of the TWEAK/Fn14 system are explored. Until now, only a recent report indicating a therapeutic modulation of this system has been published. In this work, the authors have demonstrated that treatment with hydroxy-methyl-glutaryl Coenzyme A reductase (HMG-CoA reductase) inhibitors or statins reduce Fn14 expression elicited by proinflammatory cytokines in human SMCs (15).

Statins have a great impact in the treatment of cardiovascular diseases. These drugs are selective inhibitors of the HMG-CoA reductase, a key enzyme in cholesterol biosynthesis pathway (41). Multiple clinical trials have shown that statins lower cardiovascular

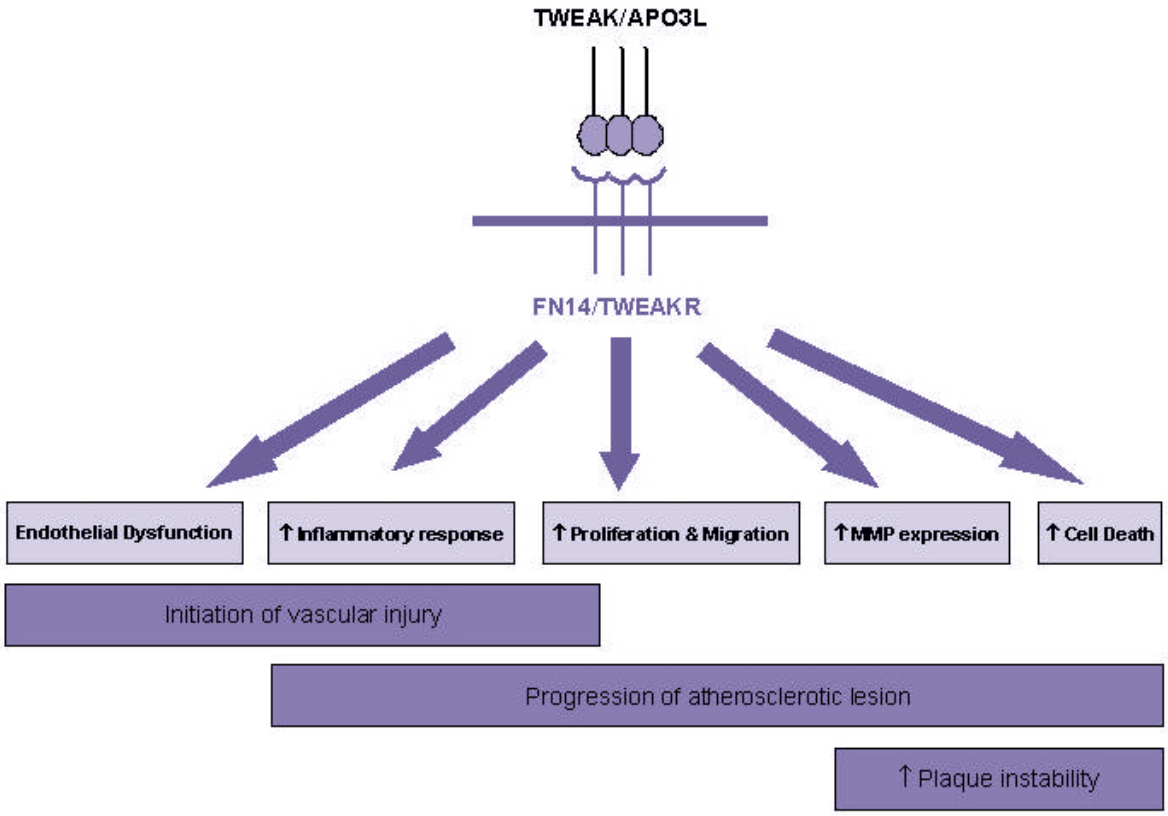

Figure 4. Schematic representation of the potential proatherogenic actions induced by TWEAK/Fn14 interaction.

morbidity and mortality, both in primary and secondary prevention (42). Statins treatment reduces cholesterol synthesis and circulating low density lipoprotein (LDL) levels, an atherogenic lipoprotein directly implicated in atherosclerosis development. Furthermore, statin treatment has also lipid-lowering independent effects, probably related with the inhibition of small G proteins prenylation (43). In this sense, Fn14 downregulation induced by atorvastatin was reversed by mevalonate, the metabolite that is directly synthesized by the HMG-CoA reductase. This suggests that metabolites from the cholesterol pathway downstream of mevalonate such as isoprenoids could be important for the regulation of Fn14. Isoprenoids participates in the posttranslational modification of important cell proteins including small G proteins (Ras and Ras-like proteins: Rac, Rab and Rho) (44). Different strategies have been demonstrated that the diminution of Fn14 expression by statins is a consequence of the inhibition of prenylation and activation of Rho protein (15). A specific inhibitor of Rho kinase also decreased Fn14 expression, indicating that the Rho/ROCK pathway may regulate Fn14 expression. Fn14 downregulation by atorvastatin had functional consequences, since this drug also decreased the proinflammatory response induced by its ligand, TWEAK, in human SMCs (15). Furthermore, treatment with statins can also reduce Fn14 expression in human carotid atherosclerotic plaques (15), indicating that this type of drugs effectively diminishes Fn14 expression both *in vitro* and *in vivo*.

6. CONCLUSION

The interaction between TWEAK and Fn14 has several potential proatherogenic effects in cultured cells which may be important in the pathogenesis of atherosclerosis. TWEAK induces production of proinflammatory cytokines, proliferation and migration of cells present in atherosclerotic plaques and increases the expression of MMPs that degrade the ECM (Figure 4). Furthermore, TWEAK has a proapoptotic action that could contribute to plaque instability. In addition, both TWEAK and Fn14 are localized to the human atherosclerotic plaque. Statins, an established therapy for atherosclerosis, downregulate Fn14 expression in cultured cells and human plaques and limit the proinflammatory action of TWEAK. Further studies should address the proatherogenic properties of TWEAK in animal models, as well as whether therapeutic benefits are obtained by blocking the interactions of TWEAK with its receptor or modulating their expression. This information will help us to better understand the role of this system in vascular disease.

7. ACKNOWLEDGEMENTS

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