Tumor Necrosis Factor Receptor Superfamily 14 Is Involved in Atherogenesis by Inducing Proinflammatory Cytokines and Matrix Metalloproteinases

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Abstract—Tumor necrosis factor (TNF) receptor superfamily 14 (TNFRSF14) is the cellular receptor for TNF superfamily 14 (LIGHT). Immunohistochemical staining of human carotid atherosclerotic plaques revealed a high level of expression of the TNFRSF14 in regions rich in macrophages/foam cells. To investigate the role of TNFRSF14 in the functioning of monocytes in relation to atherogenesis, we have analyzed TNFRSF14 expression levels and cellular events after stimulation of TNFRSF14 in peripheral blood monocytes or the human macrophage–like cell line, THP-1. A high level of expression of TNFRSF14 was detected in activated monocytes, in macrophages derived from monocytes, and in THP-1 cells. Concomitant activation of THP-1 cells with interferon- γ and immobilized anti-TNFRSF14 monoclonal antibody resulted in synergistic induction of proatherogenic cytokines, such as TNF- α and interleukin-8. Activation of THP-1 cells with immobilized anti-TNFRSF14 monoclonal antibody induced expression of matrix metalloproteinase (MMP)-1, MMP-9, MMP-13, and tissue inhibitors of metalloproteinase-1 and -2. Furthermore, immunohistochemical staining of atherosclerotic plaques with severe infiltration of foam cells revealed that the expression patterns of TNFRSF14 and MMP-1, -9, and -13 overlapped. Treatment of THP-1 cells with soluble LIGHT also caused induction of MMP-9 and interleukin-8. These data suggest that TNFRSF14 is involved in atherosclerosis via the induction of proatherogenic cytokines and decreasing plaque stability by inducing extracellular matrixdegrading enzymes. (*Arterioscler Thromb Vasc Biol.* 2001;21:2004-2010.)

> Key Words: atherosclerosis ■ immunity ■ tumor necrosis factor receptor superfamily 14 ■ matrix metalloproteinases ■ foam cells

Tumor necrosis factor (TNF)- α and CD40L play pivotal **I** roles in the atherogenesis. TNF- α was found to be expressed in atherosclerotic plaques,^{1,2} and TNF- α was also found to be colocalized with foam cells, smooth muscle cells (SMCs),^{3,4} and mast cells.⁵ CD40, a member of the TNF receptor superfamily (TNFRSF), is an integral membrane protein found on the surface of B lymphocytes, dendritic cells, hematopoietic progenitor cells, epithelial cells, and carcinomas. CD40 binds to a ligand (CD40L) which is a member of the TNF superfamily (TNFSF).6 In atherosclerotic plaques, the expression of CD40L in T cells and the coexpression of CD40 and CD40L in vascular endothelial cells, SMCs, and macrophages were detected.7 The interaction between CD40 and CD40L, similar to the interaction between TNF- α and its receptor, elicits diverse biological responses involved in atherosclerosis, such as the secretion of proinflammatory cytokines and matrix metalloproteinases

(MMPs), and the expression of adhesion molecules and tissue factor.^{8,9} These responses are known to make the plaque unstable.

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Recently, the list of molecules belonging to TNFRSF has expanded significantly. TNFRSF14 (HVEM/HveA/LIGHTR/TR2/ATAR) was initially identified as a cellular coreceptor for herpes simplex virus entry, hence, the name HVEM (herpes virus entry mediator, later named HveA [herpes virus entry protein A]).¹⁰ TNFRSF14 has a wide tissue distribution and is prominently expressed by cells in lymphoid tissue, such as the spleen, and on peripheral blood leukocytes. TNFRSF14 mRNA was detected on resting and activated CD4+ and CD8+ T cells, on CD19+ B cells, and on monocytes.^{11,12} Like other TNFRSF members, stimulation of TNFRSF14 induces activation of the

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transcription factors nuclear factor- κB and activator protein-1.^{12,13} Interestingly, TNFRSF14/TNFSF14 has cross-reactivity with lymphotoxin β -receptor/ lymphotoxin- α , linking TNFRSF14/TNFSF14 to the lymphotoxin cytokine receptor system.¹⁴

We hypothesized that TNFRSF14, like the CD40/CD40L system, has a role in atherosclerosis. We analyzed the expression of TNFRSF14 in atherosclerotic plaques and the expression of proatherogenic cytokines and MMPs after stimulation of TNFRSF14 in THP-1 cells.

Methods

Histological Analysis

For immunohistochemical analysis, carotid endarterectomy specimens were obtained from 13 patients, aged 63 to 81 years, who underwent the surgery at Samsung Seoul Hospital. The study was approved by an institutional review committee, and the subjects gave informed consent. Atherosclerotic plaque specimens were washed with saline and embedded in OCT (Miles Laboratories) to make frozen sections. Standard $5-\mu m$ sections were stained by use of the LSAB kit (DAKO) according to the manual provided by the manufacturer. Double staining of CD68 and TNFRSF14 was performed by using an Animal Research Kit (DAKO) according to the manual provided by the manufacturer.

Cell Culture

Human monocytic leukemia THP-1 cells¹⁵ were obtained from the American Type Culture Collection. For the analysis of peripheral blood monocytes, whole blood was collected either in heparin Vacutainer or CTAD Diatubes (Becton Dickinson/Diagnostica Stago) containing dipyridamole and theophylline to prevent in vitro platelet activation. Peripheral blood monocytes were isolated by using gradient centrifugation with Lymphoprep (Nycomed Pharma As), as described previously.¹⁶ To obtain macrophage-like cells from peripheral blood monocytes, isolated pure monocytes (>95% positive for CD14 staining) were incubated in RPMI medium supplemented with 10% heat-inactivated (at 68°C for 1 hour) FBS for 3 days, after which the medium was changed, and incubation was continued for 4 additional days.

Monoclonal Antibodies

Ascitic fluid of anti-TNFRSF14 monoclonal antibody (clone 139, IgG1) was generated as described by Brodeur et al.¹⁷ As a control antibody, ascitic fluid of anti-TNFRSF11B monoclonal antibody (IgG1) prepared by the same method was used. TNFRSF11B is not expressed in THP-1 cells. Possible endotoxins in the fluid were removed by Affi-prep polymixin matrix (Bio-Rad) according to the manufacturer's instructions. Endotoxin levels in the fluid were <20 pg/mL (tested with the QCL-1000 Chromogenic Limulus Amebolyte Lysate test method, Bio-Whittaker). Monoclonal antibodies to CD68 (KP1, M0814), and SMC α-actin (1A4, M0851) were purchased from DAKO. Monoclonal antibodies to HLA-DR (G46-6, 555810) were purchased from Pharmingen. Anti-MMP-1, -9, and -13 monoclonal antibodies (MAB1346, MAB13416, and MAB3321, respectively) were purchased from Chemicon International, Inc), and anti-tissue inhibitor(s) of metalloproteinase (TIMP)-1 and TIMP-2 antibodies (IM32L and IM11L, respectively) were purchased from Calbiochem.

Flow Cytometric Analysis

Flow cytometric analysis of lymphoid cells was performed on FACS-vantage (Becton-Dickinson). Whole blood was diluted 10fold with RPMI medium without serum and incubated in a CO₂ incubator in the presence of 2 ng/mL TNF- α (R&D Systems) or 1 μ g/mL lipopolysaccharide (LPS, Sigma-Aldrich,). After incubation, 1 mL of cell suspension was pelleted, and the cells were sequentially incubated with ×50 dilution of ascites containing antibody specific for TNFRSF14, 0.5 μ g of FITC-labeled rat anti-mouse IgG, and 0.5 μ g of phycoerythrin-labeled anti-CD14 antibody (MHCD1404, Caltag Laboratories) according to a previously described method.¹⁸ The fluorescence profile of 3×10^4 cells was collected, and CD14-positive cells were analyzed for the expression of TNFRSF14.

Activation of Cells, ELISA, and Gelatin Zymography

Monoclonal antibodies were diluted in PBS, and 50- μ L volumes were added to the wells of a 96-well plate and incubated at 4°C overnight. The wells were washed with PBS, and THP-1 cells (1×10⁵ cells in 100 μ L of serum-free RPMI 1640 medium) were added in each well. For TNFSF14 (LIGHT)-mediated activation, recombinant soluble human LIGHT (rhsLIGHT, Alexis) was added in 1- to 1000-ng/mL concentrations. The supernatants were collected 24 or 48 hours after activation. Cytokines were measured by a sandwich ELISA (Endogen Inc). The detection limits were <3 pg/mL for MCP-1, <2 pg/mL for interleukin-8 (IL-8), and <5 pg/mL for TNF- α . The MMP-9 activity in the culture supernatant was determined by performing substrate gel electrophoresis as described by Birkedal-Hansen and Taylor.¹⁹

Western Blot Analysis

For the detection of MMPs, culture supernatants were collected 48 hours after activation, and proteins were precipitated with cold ethanol (final concentration 60%) at -70°C for 30 minutes. Pelleted proteins (12 000g for 20 minutes) were washed with 1 mL ethanol and resuspended in PBS. Western blot analysis was performed as described previously.²⁰ For the detection of TNFSF14, 20 µg of tissue lysate was loaded in each lane for SDS-PAGE. After it was blotted onto nitrocellulose membrane (Schleicher & Schuell), the membrane was incubated in a blocking solution (5% nonfat dried milk, 1 µg/mL anti-human IgG [AI3000, Vector Laboratories Inc], and 0.1% Tween 20 in Tris-buffered saline [TBS]) for 1.5 hours, incubated overnight at 4°C with 50 ng/mL rhHVEM:Fc (522-017-C050, Alexis Corp) in TBS containing 0.1% Tween 20 (TBS-T), washed 3 times with TBS-T at 15-minute intervals, incubated for 1 hour with 1 µg/mL biotinylated anti-human IgG (BA3000, Vector Laboratories Inc) at room temperature, washed 3 times with TBS-T at 7-minute intervals, incubated for 30 minutes with horseradish peroxidase-conjugated streptavidin (DAKO) at room temperature, and washed again 3 times with TBS-T at 7-minute intervals. Bands were visualized by using enhanced chemiluminescence detection reagents (Amersham) and by exposure to x-ray films.

Results

Immunohistochemical analysis of atherosclerotic plaques obtained from carotid endarterectomy tissue revealed the expression of TNFRSF14 in 11 (84%) of 13 plaques tested. Most of the TNFRSF14 expression was detected in the shoulder region of the plaque, an area rich in foam cells and HLA-DR-positive cells (Figure 1A through 1E and 1G through 1K). Double immunohistochemical analysis further confirmed that CD68-positive foam cells are also positive for the expression of TNFRSF14 (Figure 1F and 1L). Expression of TNFRSF14 was not detected in the normal human aorta (data not shown). We then tested the expression of TNFSF14 in atherosclerotic plaques by Western analysis with the use of recombinant antibody containing an extracellular portion of TNFRSF14. Tissue lysates were obtained from regions with a thin fibrous cap and high infiltration of inflammatory cells (atheromatous region) and from regions with a thick fibrous cap and a low number of inflammatory cells (fibrous region). As expected, expression levels of TNFSF14 were higher in atheromatous regions than in fibrous regions of the plaques. The observed molecular mass of TNFRSF14 was ≈26.5 kDa, as expected.

To find out what stimulated the expression of TNFRSF14 in monocytes, human peripheral blood monocytes were analyzed



Figure 1. Expression of TNFSF14/TNFRSF14 in atherosclerotic plaques and immunohistochemical analysis of TNFRSF14. The shoulder region of a human carotid endarterectomy specimen was stained with eosins (A and G), monoclonal antibody to foam cell–specific marker, CD68 (B and H), SMC-specific marker, α -actin (C and I), TNFRSF14 (D and J), and HLA-DR (E and K) and counterstained with autohematoxylin. To confirm that TNFRSF14-positive cells are foam cells, a new section from another plaque was double-stained for CD68, which is present in late endosomes (a brown color), and TNFRSF14, which is present on the cell surface (a blue color, F and L). Low-magnification (magnification ×40) pictures are shown in panels A through F. High-magnification images of the foam cell–rich regions illustrated by the high-magnification pictures. Panel M shows Western analysis of TNFSF14 with use of tissue lysates isolated from the atheromatous region (lane 1) and fibrous region (lane 2) of a plaque. TNFSF14 and light and heavy chains of IgG were detected because anti-human IgG was used as a secondary antibody. The experiment was repeated twice with 2 separate sets of samples, with essentially the same results.

after activation of total human blood with various stimuli. Incubation of peripheral blood isolated from 3 normal healthy subjects for 2 hours did not induce any increase in the basal level of TNFRSF14, whereas the addition of TNF- α induced statistically significant induction of TNFRSF14 in monocytic cells (Figure 2A). Concomitant increase in the monocyte activation marker, CD14, was observed. Incubation of peripheral blood for 6 hours induced an increase in basal expression level of TNFRSF14, and treatment with either TNF- α (data not shown) or bacterial LPS (Figure 2B) induced further increase in the TNFRSF14 expression level. We then isolated peripheral blood monocytes and incubated these cells for a week to induce macrophage differentiation.^{21,22} High-level expression of TNFRSF14 was observed in these cells (Figure 2C).

It has become common to activate cellular cytokine receptors by cross-linking them to immobilized antibodies. Antibodies to the type I TNF receptor have been shown to trigger a variety of TNF-like effects on cross-linkage of the receptor molecules.²³ Furthermore, cross-linking CD40 to immobilized anti-CD40 can induce the proliferation of B cells and the secretion of IgE after isotype switching.⁶ These events were originally known to be induced in B cells activated by T cells expressing CD40L. We then tested the cellular responses mediated by TNFRSF14. Concomitant treatment of THP-1 cells, which express high

basal levels of TNFRSF14, with immobilized anti-TNFRSF14 monoclonal antibody and 100 U/mL interferon (IFN)- γ resulted in the induction of TNF- α and IL-8 secretion (Figure 3A and 3B, respectively). Activation of THP-1 cells with immobilized anti-TNFRSF14 antibody or IFN- γ alone induced only low levels of TNF- α or IL-8. Treatment of THP-1 cells with rhsLIGHT resulted in activation of IL-8 expression (Figure 3C).

Stimulation of the TNFRSF14 by immobilized monoclonal antibody induced the expression of MMP-9 in a time- and dose-dependent manner, as observed in a gelatin zymogram (Figure 4A). When THP-1 cells were treated with rhsLIGHT, induction of MMP-9 was also detected (Figure 4B). Induction of MMP-9 was confirmed by Western blot analysis (Figure 4C). Because MMP-1 has been reported to be expressed in THP-1 cells activated with LPS, we tested whether MMP-1 and MMP-13 are induced by the stimulation of TNFRSF14. As shown in Figure 4D, MMP-1 and MMP-13 were induced by treatment of THP-1 cells with immobilized anti-TNFRSF14 monoclonal antibody. Activation of THP-1 cells with immobilized anti-TNFRSF14 monoclonal antibody also resulted in the activation of TIMP-1 and TIMP-2 (Figure 4E).

Because MMPs and TIMPs have been shown to be inducible by stimulation with TNFRSF14, we tested



Figure 2. Activation of peripheral blood monocytes results in upregulation of TNFRSF14. A, Human peripheral blood was treated with 2 ng/mL TNF- α for 2 hours, and monocyte expression of TNFRSF14 and CD14 was tested by flow cytometry as described in Methods. Mean fluorescence intensity (MFI) values obtained from 3 different individuals were compared. Open, hatched, and solid bars represent the MFI of specific staining after TNF- α treatment (+), with no treatment (-), and with background staining (Cont), respectively. The difference between TNFRSF14 MFI levels with or without TNF- α addition was tested by nonparametric t test (Mann-Whitney test). *P < 0.05; ***P<0.001. B, Human peripheral blood was treated with 1 μ g/mL LPS for 6 hours, and monocyte expression of TNFRSF14 and CD14 was tested. Filled areas represent TNFRSF14-specific fluorescence, and the open area represents the background level of fluorescence given by the secondary antibody. C, Peripheral blood monocytes were incubated for 1 week to induce macrophage differentiation and were tested for the expression of TNFRSF14.

whether the expression pattern of these molecules overlapped in atherosclerotic lesions. TNFRSF14 was expressed in foam cell–rich regions in the atherosclerotic plaque, as were MMP-1, MMP-9, and MMP-13, but neither TIMP-1 nor TIMP-2 was detected in similar regions (Figure 5). We tested 7 additional plaques for the expressions of MMPs and TIMPs in relation to TN-FRSF14. In general, the distribution pattern of foam cells



and TIMPs overlapped in plaques with a mild infiltration of foam cells (data not shown). However, in severe plaques with heavy foam cell infiltration (Figure 5), TIMPs either were expressed at a low level or were absent.

Discussion

Our results indicate that the TNFRSF14 may be a mediator of atherosclerosis by inducing proatherogenic cytokines and MMPs.

Upregulation of the TNFRSF14 and CD14 expression levels in peripheral blood monocytes incubated with TNF- α or LPS indicates that activation of monocytes results in concomitant upregulation of TNFRSF14 and CD14, the monocyte activation marker. This is further supported by expression of TNFRSF14 in macrophages derived from peripheral blood monocytes. Monocytes cultured for 7 days with 10% autologous serum were reported to be spontaneously differentiated into macrophage-like cells expressing macrophage-specific CD68 antigen and high basal levels of tissue factor activity, a property unique to mature macrophages.^{21,22} Monocytes/ foam cells present within the plaque are under stimulation by various agents, including oxidized LDL, proatherogenic cvtokines, and cell-to-cell contact with activated T lvmphocytes. These agents could be responsible for the expression of TNFRSF14 in activated monocytes. Our histological data indicate that the cells expressing TNFRSF14 in atherosclerotic plaques are monocytes/foam cells. Observations showing expression of TNFRSF14 in activated peripheral blood monocytes, in macrophage-like cells derived from peripheral blood monocytes by long-term culture, and in the macrophage-like cell line THP-1 further support the conclusion that it is the foam cells in the plaque that express TNFRSF14.

TNFRSF14 expressed on the surface of foam cells in the plaque is likely to be stimulated by cells expressing TNFRSF14 ligand. The ligand for TNFRSF14 is known to be TNFSF14 (LIGHT). TNFSF14 mRNA was detected in spleen cells, activated peripheral blood lymphocytes,

> Figure 3. Activation of TNFRSF14 induces expression of proatherogenic cytokines in THP-1 cells. A, THP-1 cells (1×10⁵ per well in a 96-well plate) were stimulated with 1 µg/mL LPS or immobilized monoclonal antibodies with (solid bar) or without (open bar) the addition of IFN-y (100 U/mL). Supernatants were collected 48 hours after activation, and the expression level of TNF- α was measured by ELISA. Flowcytometric analysis of TNFRSF14 expression level in THP-1 cells is shown in the inset. The filled area represents TNFRSF14-specific fluorescence, and the open area represents the background level of fluorescence given by the secondary antibody. Control Ab indicates control antibody. B, THP-1 cells (1×10⁵ cells per well in a 96-well plate) were stimulated with 1 µg/mL LPS or immobilized monoclonal antibodies with (solid bar) or without (open bar) the addition of IFN-y (10 U/mL). Supernatants were collected 24 hours after activation, and the level of IL-8 was measured by ELISA. C, THP-1 cells (2×10⁵ cells per well in a 96-well plate) were stimulated with 1 µg/mL LPS or rhsLIGHT with (solid bar) or without (open bar) the addition of IFN-y (10 U/mL). Supernatants were collected after 24 hours, and the level of IL-8 was measured by ELISA.





CD8⁺ tumor-infiltrating lymphocytes, granulocytes, and monocytes. TNFSF14 is also expressed by activated T lymphocytes and macrophages.²⁴ It is likely that T lymphocytes and macrophages that are present in the atherosclerotic plaque express TNFSF14, which could stimulate TNFRSF14-mediated inflammatory reactions in foam cells. Our data (Figure 1M) confirm that TNFSF14 is expressed in atherosclerotic plaques, and the expression levels were higher in regions with inflammation.

IFN- γ has been reported to be expressed in atherosclerotic plaques and to regulate the expression of macrophage scavenger receptors.²⁵ IFN- γ also proved to be involved in atherogenesis, by acting on SMCs to potentiate growth factor–induced mitogenesis.²⁶ Our data indicate that the concomitant treatment of THP-1 cells with TFN- γ and immobilized anti-TNFRSF14 monoclonal antibody causes the synergistic activation of proatherogenic cytokines. This raises the possibility that signaling mediated by TNFRSF14 could work in concert with IFN- γ to induce the further secretion of proatherogenic cytokines, such as TNF- α and IL-8, in atherogenesis.

The stability of atherosclerotic plaque depends on the integrity of the fibrous cap, which, in turn, depends on the content of extracellular matrix protein. Dysregulation between MMPs and their inhibitors, TIMPs, is believed to be responsible for the rupture of atherosclerotic plaques.²⁷ MMP-1 and MMP-13, which degrade fibrillar collagen, were found to be expressed in foam cell–rich regions in atheromatous plaque.²⁸ MMP-9, which degrades nonfibril-

lar collagen, is also known to be expressed in atherosclerotic plaques.^{29,30} As shown in Figure 5, expression of MMP was detected in the shoulder region of atherosclerotic plaque, and the expression pattern overlapped the expression of TNFRSF14.

It is interesting that the expressions of TIMP-1 and TIMP-2 were observed in THP-1 cells after stimulation of TNFRSF14 but that TIMP expression was not detected in the plaque. This could be because the differentiation stages of THP-1 cells and of foam cells in the plaque are different. It is also possibly due to differences in the activation signal, inasmuch as THP-1 cells received an activation signal only via TNFRSF14 in our in vitro experiment, whereas foam cells in the plaque received multiple signals in addition to the signal via TNFRSF14.

Our data suggest that TNFRSF14 is involved in atherosclerosis via the induction of proatherogenic cytokines and the decrease in plaque stability by inducing extracellular matrix–degrading enzymes. Because these functions have also been linked to other cytokines, such as TNF- α and CD40/CD40L,^{1,3,5–8} it can be expected that TNFRSF14 and TNFSF14 are contributing factors in atherosclerosis. Further investigations are required to determine whether blocking the interaction between TNFRSF14 and TNFSF14 could suppress the atherogenic process.

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Figure 5. Immunohistochemical analyses of consecutive sections of a human carotid endarterectomy specimen reveled correlation between the expression of TNFRSF14 and MMPs. Shoulder regions were stained with no primary antibody (A), primary antibodies specific for CD68 (B), TNFRSF14 (C), HLA-DR (D), MMP-1 (E), MMP-9 (F), MMP-13 (G), and TIMP-2 (H). Bottom panels are high magnification (\times 400) of the view of the upper panels (magnification \times 40). Square in panel A indicates region where high-magnification pictures were taken. L indicates lumen; M, media.

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