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Tumor Necrosis Factor Receptor Superfamily 12 may Destabilize Atherosclerotic Plaques by Inducing Matrix Metalloproteinases

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Immunohistochemical staining of human atherosclerotic plaques revealed expression of the tumor necrosis factor receptor superfamily (TNFRSF) 12 in regions rich in macrophage/foam cells. The role of TNFRSF12 in the functioning of monocytes in relation to atherogenesis was investigated by analysis of cellular events after stimulation of TNFRSF12 in a human macrophage-like cell line, THP-1. Activation of the THP-1 cells on plates coated with monoclonal antibody against TNFRSF12 induced the expression of matrix metalloproteinases (MMPs) -1, -9, and -13. Furthermore, the expression patterns of TNFRSF12 and the MMPs overlapped in atherosclerotic plaques. Signaling of TNFRSF12 may thus contribute to the induction of extracellular matrix degrading enzymes in macrophages. (*Jpn Circ J* 2001; **65:** 136–138)

Key Words: Atherosclerosis; Matrix metalloproteinase; Tumor necrosis factor receptor superfamily

The stability of atherosclerotic plaques depends greatly on the integrity of the fibrous cap, which in turn depends on its extracellular matrix protein content. Dysregulation of the interplay between matrix degrading enzymes, such as matrix metalloproteinases (MMPs), and their inhibitors, such as tissue inhibitor of metalloproteinases (TIMPs), is believed to be responsible for the rupture of atherosclerotic plaques[1] MMP-1 and MMP-13, which degrade fibrillar collagen, are expressed in foam cell rich regions in atheromatous plaques[2] MMP-9, which degrades non-fibrillar collagen, is also known to be expressed in atherosclerotic plaques[3]

Tumor necrosis factor receptor superfamily (TNFRSF) 12 (DR3) contains a death domain similar to tumor necrosis factor receptor-1 (TNFR-1) and CD95 (also called Fas or APO-1), hence the name DR3 (death receptor 3). Expression of DR3 appears to be restricted to tissues enriched with lymphocytes. The signaling through DR3 induces both apoptosis and activation of nuclear factor (NF)-κB[4]

Tumor necrosis factor (TNF)-α and CD40L, members of the TNF superfamily, participate in atherogenesis by inducing pro-inflammatory cytokines, chemokines, MMP and tissue factor (TF) expression[5,6] All these cellular responses and factors are known to make plaques unstable. We hypothesized that TNFRSF12, as with TNF-α and CD40/CD40L, has a role in atherosclerosis and so we analyzed its expression in atherosclerotic plaques. Subsequently, we looked into the expression of MMPs in THP-1 cells (American Type Culture Collection, Rockville, MD, USA) after stimulation of the TNFRSF12.

The immunohistochemical analysis of human atherosclerotic plaques obtained from carotid endoarterectomies was approved by an institutional review committee and the subjects gave their informed consent. Standard 5-mm frozen sections were stained using the LSAB kit (DAKO, Copenhagen, Denmark). Ascites fluid derived from mouse hybridoma cells expressing monoclonal antibody (mAb) against recombinant human TNFRSF12 was used for immunohistochemistry. The distribution pattern of TNFRSF12 positive cells (Fig 1C) overlapped with the presence of foam cells (Fig 1B), which were identified by staining with mAb to a foam cell specific marker, CD68 (KP1, DAKO). Expression of TNFRSF12 was detected in 82% of plaques tested (9 of 11 patients, aged 63–81, who underwent surgery at Samsung Seoul Hospital).

It has become common use to activate cellular cytokine receptors by cross-linking them to immobilized antibodies. Antibodies to type I TNF receptor have been shown to trigger a variety of TNF-like effects upon cross-linkage of the receptor molecules[7] Furthermore, cross-linking CD40 to immobilized anti-CD40 can induce B cells to proliferate and secrete IgE following isotype switching[8] These events were originally known to be induced by the activation of B cells by activated T cells expressing CD40L. Flow cytometry using anti-TNFRSF12 mAb revealed that the THP-1 cells expressed high basal levels of TNFRSF12 (Fig 2A). We then tested for cellular responses when TNFRSF12 was activated in these cells using plate-bound anti-TNFRSF12 mAb. To coat the plates, anti-TNFRSF12 mAb was diluted in phosphate buffered saline (PBS) and added in a volume of 50 μl/well to a 96-well plate, which was then incubated at 4°C overnight. After a thorough washing of the wells, THP-1 cells (1×10^5 cells in 100 μl of serum-free RPMI1640 medium) were added. Culture supernatants were collected

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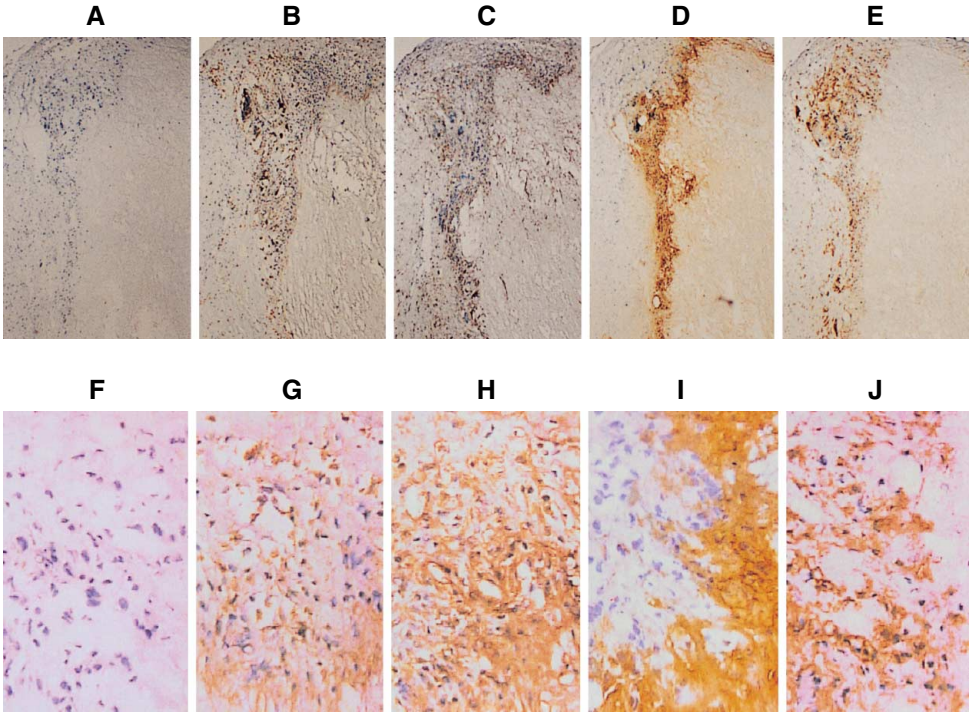

Fig 1. Immunohistochemical analysis of atherosclerotic plaques obtained from human carotid endoarterectomy. Frozen sections were stained without the addition of primary antibody (A and F) and with the addition of monoclonal antibody to foam cell specific marker CD68 (B and G), anti-TNFRSF12 (C and H), anti-MMP-9 (D and I), and anti-MMP-13 (E and J). (F–J are high magnifications (×200) of the view of the foam cell rich regions in A–E, respectively (×40).

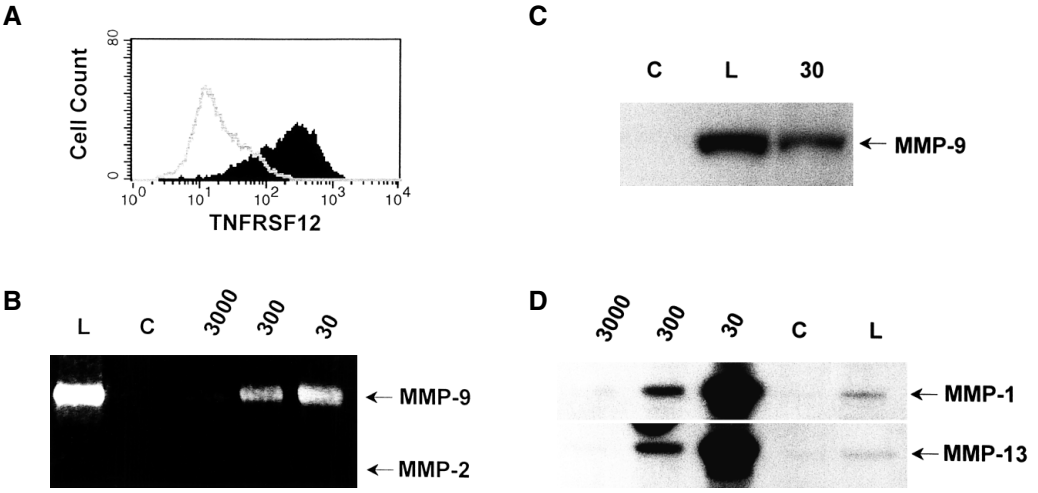

Fig 2. Activation of TNFRSF12 induces expression of MMPs in THP-1 cells. (A) Flow-cytometric analysis of TNFRSF12 expression in THP-1 cells. Filled area represents TNFRSF12 specific fluorescence and empty area represents background levels of fluorescence caused by the secondary antibody. (B) Gelatin zymogram of THP-1 culture supernatants. Cells were stimulated without (*C*) or with 1 mg/ml lipopolysacuharide (LPS) (*L*) or by incubation with plate-bound anti-TNFRSF12 monoclonal antibody. Numbers indicate dilution factors of the ascitic fluids containing the monoclonal antibodies. (C, D) THP-1 cells were activated with LPS or plate-bound anti-TNFRSF12 monoclonal antibody for 48 h, and production of MMP-9 (C), MMP-1, and MMP-13 (D) was determined by Western blot analysis.

after 24-h incubation. MMP-9 activity in the culture supernatant was determined by substrate gel electrophoresis as described by Birkedal-Hansen and Taylor[9] Data revealed that the stimulation of TNFRSF12 resulted in the induction of MMP-9 expression (Fig 2B), which was confirmed by Western blot analysis (Fig 2C).

Because MMP-1 is reportedly expressed in activated THP-1 cells,[10] we also tested whether MMP-1 and -13 were induced upon stimulation of TNFRSF12. As shown in Fig 2D, MMP-1 and MMP-13 were induced by the treatment of THP-1 cells with plate-bound anti-TNFRSF12 mAb. For the Western blot analysis of the MMPs, culture supernatants

were collected 48 h after activation, and the proteins were precipitated with cold ethanol (final concentration 60%) at –70°C for 30 min. Pelleted proteins (12,000 G for 20 min) were washed with 1 ml of ethanol and resuspended in PBS. The Western blot was probed with ×1000 diluted anti-MMP-1, -9, or -13 mAb (Chemicon International, Inc, Temecula, CA, USA).

Because we observed that MMP-1, -9, and -13 could be induced by stimulating TNFRSF12, we investigated the expression patterns of these molecules in atherosclerotic lesions and found that TNFRSF12 (Fig 1C), MMP-1 (data not shown), MMP-9 (Fig 1D), and MMP-13 (Fig 1E) exhibited a similar pattern.

Our results suggest that TNFRSF12 could be a novel mediator of atherosclerosis by inducing MMPs. Monocytes/foam cells within the plaque are under stimulation by various agents, including oxidized-low density lipoprotein and pro-atherogenic cytokines, and cellular interactions with activated T-lymphocytes. These agents could be responsible for the expression of TNFRSF12 on activated monocytes.

The TNFRSF12 expressed by foam cells in the plaque is likely to be activated by a TNFRSF12 ligand, which is known to be TWEAK (TNFSF12) and has a wide tissue distribution and is efficiently secreted from cells![11] Our data suggest that further work be done to find and confirm the distribution pattern of the natural ligands for TNFRSF12, including TWEAK, within plaques.

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