TWEAK Can Induce Pro-Inflammatory Cytokines and Matrix Metalloproteinase-9 in Macrophages

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The expression of TWEAK (TNFSF12) and TweakR/Fn14 was detected in regions rich in macrophage/foam cells in atherosclerotic plaques. The role of TWEAK in monocytes in relation to atherogenesis was investigated by analyzing the cellular events induced by TWEAK in a human macrophage-like cell line, THP-1. TWEAK induced various molecular mediators of atherogenesis, such as IL-6, MCP-1, IL-8 and MMP-9, and the induction was augmented by interferon-\( \gamma \). TWEAK-induced activation of MMP-9 was mediated by activation of NF-\( \kappa B \). These results suggest that TWEAK is involved in atherosclerosis by inducing pro-inflammatory cytokines and extracellular matrix degrading enzymes, which reduce plaque stability. (Circ J 2004; 68: 396–399)

Key Words: Atherosclerosis; Foam cells; TWEAK

Interaction between members of the tumor necrosis factor superfamily (TNFSF) and their receptors (eg, TNF-\( \alpha \), CD40/CD40L and LIGHT/TR2) elicit diverse biological responses that are known to be involved in atherogenesis. These responses include the induction of pro-inflammatory cytokines, matrix metalloproteinases (MMPs), adhesion molecules, and tissue factor, and are known to make plaque unstable. TWEAK (TNFSF12) has multiple biological activities, including stimulation of cell growth, induction of inflammatory cytokines, and stimulation of apoptosis. The receptor for TWEAK is known to be TweakR/Fn14. The expression of TWEAK is induced in peripheral blood monocytes by interferon-gamma (IFN-\( \gamma \)). Because IFN-\( \gamma \) expression has been detected in atherosclerotic plaques, it is expected that TWEAK is expressed by macrophage cells in atherosclerotic plaques. Therefore, we investigated the expression of TWEAK in atherosclerosis and the possible cellular responses induced by TWEAK.

Human carotid endarterectomy tissues were obtained from 13 patients (aged 63–81 years, who underwent surgery at the Samsung Seoul Hospital) after informed consent was given. Immunohistochemical analysis of the atherosclerotic plaques revealed TWEAK and TweakR/FN14 expressions in regions rich in foam cells (Fig 1, Top panel). Foam cells, as detected by CD68 staining, were found to be distributed in necrotic cores and the shoulder regions of the plaques. Double staining for TWEAK or TweakR and CD68 for macrophages/foam cells (Fig 1, Middle and Bottom panels) confirms that foam cells express TweakR. For historical analysis, standard 5-\( \mu \)m sections were stained using an LSAB kit (DAKO, Glostrup, Denmark) according to the manual provided by the manufacturer. Monoclonal antibody to TWEAK (generously provided by Dr Yagita of Juntendo University), CD68 (DAKO), and TweakR/FN14 (eBioscience, San Diego, CA, USA) were used.

IFN-\( \gamma \) has been reported to be expressed in atherosclerotic plaques and to regulate the expression of the macrophage scavenger receptor. IFN-\( \gamma \) was also found to be involved in atherogenesis, by acting on smooth muscle cells to potentiate growth-factor-induced mitogenesis and is known to induce the expression of TWEAK in peripheral blood monocytes. Thus, the expression of TWEAK in foam cells in atherosclerotic plaques is likely to be induced by IFN-\( \gamma \) in combination with other atherogenic stimuli. TWEAK expressed by foam cells in atherosclerotic plaques will autostimulate itself through TweakR/FN-14.

Flow cytometry using anti-TweakR/FN14 monoclonal antibody revealed that the THP-1 cells (ATCC, Rockville, MD, USA) expressed a high basal level of TweakR/FN14 (Fig 2A). To find out the cellular responses induced by TWEAK, we treated THP-1 cells with recombinant human soluble TWEAK protein (rhsTWEAK; Alexis, San Diego, CA, USA). Stimulating THP-1 cells with rhsTWEAK induced the expression of MMP-9 in a dose-dependent manner, as observed in a gelatin zymogram (Fig 2B). Treatment of THP-1 cells with rhsTWEAK induced pro-atherogenic cytokines, such as IL-6, IL-8, and MCP-1. Interestingly, the treatment of THP-1 cells with 100 U/ml of IFN-\( \gamma \) in combination with 100 or 300 ng/ml of TWEAK resulted in the synergistic activation of IL-8, IL-6, and MCP-1 (Fig 2C). For the assay, THP-1 cells (1×10^5/well) were seeded in 100-\( \mu \)l of serum-free RPMI1640 medium in 96-well plates. The supernatants were collected 24 or 48 h after activation and MMP-9 expression was measured by gelatin zymogram and cytokines were measured by sandwich ELISA (Endogen Inc, Woburn, MA, USA).

Activation of NF-\( \kappa B \) has been reported to play an important role in the activation of pro-inflammatory cytokines and MMPs; a recent study showed that TWEAK initiates...
NF-κB signaling pathways. Therefore, we investigated whether TWEAK causes NF-κB activation by electrophoretic mobility shift assay (EMSA). THP-1 cells were activated as described earlier. Nuclear extracts (10 μg) were mixed with poly(dI-dC)-poly(dI-dC) (1 μg) in 10–30 μl of binding buffer (20 mmol/L HEPES, pH 7.9, 20% glycerol, 100 mmol/L KCl, 0.2 mmol/L EDTA, 10 mmol/L DTT, and 30 mmol/L PMSF). End labeled NF-κB probe (Promega, Madison, WI, USA) (0.002–0.008 pmol/5,000 cpm) was added 5 min later, and the cells were incubated for 20 min at room temperature. Next, 1 μl of dye was added to each tube, the mixture was applied to a 4% polyacrylamide gel and electrophoresed. For supershift analysis, 1 μg of anti-p65 antibody (SantaCruz Biotechnology, San Diego, CA, USA) was added during the 20 min incubation period before electrophoresis. As shown in Fig 2D, nuclear localization of NF-κB was observed within 30 min of treating the THP-1 cells (Fig 2E). Western blot analysis was performed as described previously. To confirm that the activation of NF-κB by TWEAK is required for the induction of MMP-9, we pre-treated THP-1 cells with the NF-κB inhibitor, PDTC, before TWEAK activation. PDTC inhibited the activation of MMP-9 in a dose-dependent manner, and pretreatment with 20 μmol/L of PDTC blocked the activation of MMP-9 (Fig 2F). To rule out the possibility of cytotoxic effects of PDTC, we carried out cell viability test at 24 and 48 h after treatment with 20 μmol/L PDTC; we did not detect any decline of THP-1 cell viability after PDTC treatment (data not shown), which indicates that TWEAK-mediated activation of MMP-9 requires activation of NF-κB.

Our results suggest that TWEAK is involved in atherogenesis via the induction of pro-atherogenic cytokines and extracellular matrix degrading enzymes. These responses are known to decrease the stability of the plaques by activating other cell types and weakening the fibrous cap that...
Fig 2. TWEAK induces the expression of MMP-9 and pro-atherogenic cytokines in THP-1 cells. (A) Flow-cytometric analysis of TweakR/FN14 expression in THP-1 cells. Filled area represents TweakR/FN14 specific fluorescence and empty area represents background levels of fluorescence caused by secondary antibody. (B) Gelatin zymogram of THP-1 culture supernatants. THP-1 cells were stimulated with 1 μg/ml of LPS or rhsTWEAK, and the supernatants were collected after 24 h. (C) THP-1 cells were treated with 1 μg/ml of LPS or 30, 100, and 300 ng/ml of rhsTWEAK in the presence or absence of IFN-γ (10 or 100 U/ml), and the expression levels of IL-8, IL-6, and MCP-1 in the supernatants were measured by ELISA after 48 h of activation. (D) EMSA using a NF-κB probe and nuclear extract isolated from THP-1 cells stimulated with recombinant TWEAK. For supershift of the NF-κB band, anti-p65 monoclonal antibody was added as indicated. Nuclear extract isolated from THP-1 cells treated with 10 ng/ml TNF-α was used as a positive control. (E) Western blot analysis of cell lysates with anti-IκB monoclonal antibody. Cell lysates were isolated from THP-1 cells stimulated with 100 ng/ml of recombinant TWEAK for the indicated time. (F) THP-1 cells were stimulated with 1 μg/ml of LPS (as a positive control) or 100 ng/ml of recombinant TWEAK in the presence or absence of PDTC. Cell culture supernatants were collected after 48 h and subjected to gelatin zymography.
covers the atherosclerotic plaque. Because these functions have been substantively linked to other members of the TNFSF, it is reasonable to expect that TWEAK is a contributory factor in atherosclerosis. Further investigations are needed to determine whether blocking the interaction between TWEAK and TweakR/FN14 will suppress the atherogenic process.

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References