

Biological Activity of Tumor Necrosis Factor- α Secreted from Smooth Muscle Cell Overexpressing FADD

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This study investigated biological activity of tumor necrosis factor (TNF)- α secreted from smooth muscle cell (SMC) destined for death by expressing Fas associated death domain containing protein (FADD) (FADD-SMC) when the cells are grown without tetracycline in culture medium. In the absence of tetracycline the FADD-SMC secreted approximately 1000 pg/ml TNF- α , whereas hardly detectable amount of the cytokine existed in the presence of tetracycline. The culture medium collected from the FADD-SMC grown in the absence of tetracycline increased phosphorylated form of p38 MAPK and up-regulated nuclear factor kappa B (NF- κ B). The medium collected without tetracycline also caused death of L929 cells. Depletion of TNF- α with the soluble TNF receptor (sTNFR) inhibited the phosphorylation of p38 MAPK, the up-regulation of NF- κ B activity and the death activity of the medium collected from FADD-SMC in the absence of tetracycline. These results indicate that TNF- α secreted from SMC undergoing death is biologically active and can affect cellular function.

Key words – Apoptosis, p38 MAPK, Smooth muscle cell, Tumor necrosis factor- α

Introduction

In vasculature, death of vascular smooth muscle cell (VSMC) occurs not only in physiological arterial remodeling[8] but also in disease states including atherosclerosis and aneurysm[9,13,16]. In contrast to the notion that apoptotic cell death is essential in development and homeostasis in tissues for removal of either infected or damaged cells[21], cell death including apoptosis in VSMC is believed to be deleterious in vascular diseases. VSMC death has been proposed to contribute to plaque hypocellularity[2], blood coagulation[10], and vascular calcification[19]. VSMC death also is believed to weaken texture and reduce deposition of extracellular matrix protein in the plaque, leading to plaque instability and rupture[2,3,14]. Plaque rupture and subsequently occurring thrombosis are crucial events in acute coronary syndromes and in the progression of underlying atherosclerotic diseases[15,16]. Thus, understandings of the mechanisms and the factors associated with VSMC biology death are of importance in therapeutic strategies for vascular diseases.

FADD was identified as an adaptor molecule linking the activated Fas (CD95) receptor to the effector molecule caspase-8. It is essential for apoptotic death signaling of Fas

receptor[5] and is also involved in death induction by other death receptors of tumor necrosis factor receptor 1 (TNFR1) and DR3[1]. FADD contains a death domain (DD) at its C terminus. The DD domain binds to the cytoplasmic region of receptors. The N terminus of FADD contains a death effector domain (DED), which is essential for caspase 8 recruitment. FADD is not just an adaptor molecule in death signaling. It can activate death pathway as over-expression of FADD triggered apoptotic death in cells[6,7]. In addition to apoptosis, FADD seems to function in a number of different signaling pathways. Thymocytes and peripheral T cells expressing dominant negative form of FADD showed defect in activation-induced proliferation. FADD knockout T cells showed impaired proliferation following activation, suggesting a role for FADD in T cell development and activation[22].

Contrary to the generally held opinion that apoptosis is silent and non-inflammatory, there are reports that Fas-mediated apoptosis can trigger inflammatory reactions. FADD-induced apoptosis resulted in a massive inflammatory response[20]. Fas stimulation triggered neo-angiogenesis and local infiltration of inflammatory cells, independently of apoptosis[4]. We found up-regulation and secretion of TNF- α in dying rat SMC by expressing FADD in the absence tetracycline. Using the SMC, we investigated whether the secreted TNF- α had effects on cellular function.

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Materials and Methods

Cell culture

Rat smooth muscle cells and L929 cells were grown in Dulbecco's modified Eagle's medium-high glucose (DMEM) (Life Technology, Grand Island, NY) supplemented with 10% fetal bovine serum, 5 mM L-glutamine, plus 50 units/ml penicillin and 50 g/ml streptomycin in a humidified atmosphere of 5% CO₂. As FADD-SMCs express and undergo apoptotic death when they are cultured without tetracycline, the cells are cultured in the presence of 10 µg/ml of tetracycline in the medium.

Reagents

Antibodies for phospho-p38 MAPK was from New England Biolabs Inc. (Beverly, MA). The soluble TNF-α receptor (sTNFR) was from Immunex Corporation (Seattle, WA). Rat TNF ELISA kit was from BD Biosciences Pharmingen (San Diego, CA).

ELISA of TNF-α

The amount of TNF-α present in culture medium was determined following manufacturer's instructions. In brief, a 96 well plate was coated overnight at 4°C with capture antibody. After washing, wells were blocked for an hour at room temperature. During the blocking, standard and samples were prepared. Samples, standard, and control were transferred into appropriate well. After 2 hours at room temperature, wells were washed and incubated for an hour in the presence of diluted detection antibody, followed by enzyme reagent. After extensive washes, substrate solution was added and incubated for 30 minutes at room temperature in the dark. The absorbance was measured at 450 nm with a reference wavelength of 570 nm after addition of stop solution.

Western blot analysis

Cells were lysed in a lysis buffer (50 mM TrisCl, pH 7.8, 150 mM NaCl, 1% NP40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride). The protein content was determined using BCA Protein Assay Reagent (Pierce, Rockford, IL). Twenty micrograms of protein was separated on SDS-PAGE gels and transferred to polyvinylidene difluoride membrane (Millipore Corp.). Nonspecific binding sites were blocked in T-TBST (50 mM TrisHCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry

milk for 2 hours at room temperature. The membrane was incubated with primary antibodies in T-TBST at 4°C overnight. After 3 washes with T-BST, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody. Being washed 3-4 times with T-TBST, membrane was incubated with Enhanced Luminol Reagent. The chemiluminescent signal was imaged on the X-ray film.

Measurement of oligonucleosomes

Oligonucleosomes in cells treated with appropriate reagents were quantitatively determined with the cell-death detection ELISAPLUS kit following manufacturer's instructions (Roche, Indianapolis, IN). Cells were collected, resuspended in the lysis buffer provided in the kit, and incubated for 30 minutes at room temperature. The resultant supernatants after centrifugation at 200 g for 10 minutes were transferred into the streptavidin coated microplate with the Immunogen reagent. After incubation for 2 hours with gentle shaking (300 rpm), each well was washed three times with the incubation buffer in the kit. The developing solution was added to each well and incubated on a plate shaker at 250 rpm until the color development was sufficient for a photometric analysis. The absorbance was measured at 405 nm with a reference wavelength of 492 nm.

Results

Quantitation of TNF-α secreted by FADD-SMC

The authors have evidence that FADD-SMC secreted TNF-α into the medium when they were grown in the absence of tetracycline. To determine the amount of TNF-α secreted by the SMC, cells were incubated with or without tetracycline for indicated time period and culture medium was collected and concentrated. TNF-α in the medium was examined by ELISA after it was concentrated (Fig. 1). TNF-α was detected from FADD-SMC cultured for 2 days without tetracycline. The secretion was increased up to 3 days after tetracycline removal. The cytokine, however, was not detected in the culture medium collected from the SMC grown with tetracycline.

Phosphorylation of p38 MAPK and up-regulation of NF-κB activity by the secreted TNF-α

TNF-α is able to activate MAPKs and NF-κB in rat SMC. Thus, it was examined whether the medium collected without tetracycline activated p38 MAPK and NF-κB.

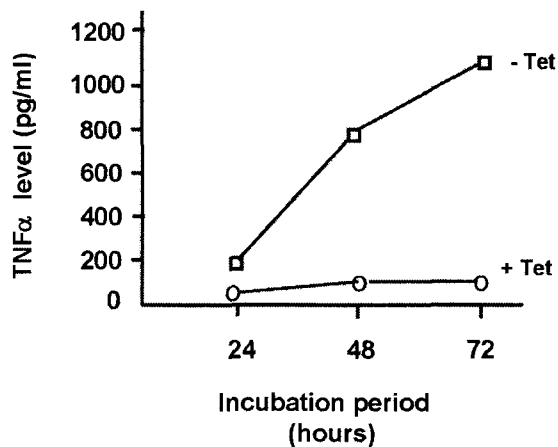


Fig. 1. Measurement of secreted TNF- α into culture medium. FADD-SMCs were cultured in the absence (-Tet) or presence (+Tet) of tetracycline. The culture medium was collected at the indicated time point and concentrated with Centricon. The amount of TNF- α in the medium was determined by ELISA. Data are the representative of three independent experiments in triplicate. Data are presented as mean of triplicate.

Normal rat SMCs were incubated with the medium collected from FADD-SMC with or without tetracycline, and phosphorylated form of p38 MAPK was detected by Western blot analysis (Fig 2). Phosphorylated form p38 MAPK was noticeably increased as early as 10 minutes after addition of the medium collected without tetracycline and sustained up to 60 minutes post incubation. To investigate contribution of the TNF- α to the phosphorylation of p38 MAPK, TNF- α in the medium was either neutralized with soluble TNF receptor (sTNFR) or incubated corresponding Fc portion of IgG (FcIgG) to which sTNFR is fused. Then phosphorylated form of p38 MAPK was detected. As a positive control, recombinant TNF- α was employed. TNF- α increased phosphorylated form of p38 MAPK, which was profoundly blocked by sTNFR. A similar pattern of inhibition was observed with the medium collected without tetracycline. The presence of sTNFR significantly blocked phosphorylation form of p38 MAPK by the medium.

Next it was investigated whether NF- κ B was influenced by the conditioned medium. Normal SMCs were transfected with NF- κ B reporter plasmids and treated with culture medium collected from FADD-SMC with or without tetracycline. Then luciferase activity was evaluated. NF- κ B activity was noticeably increased by the medium collected without tetracycline. To investigate contribution of the

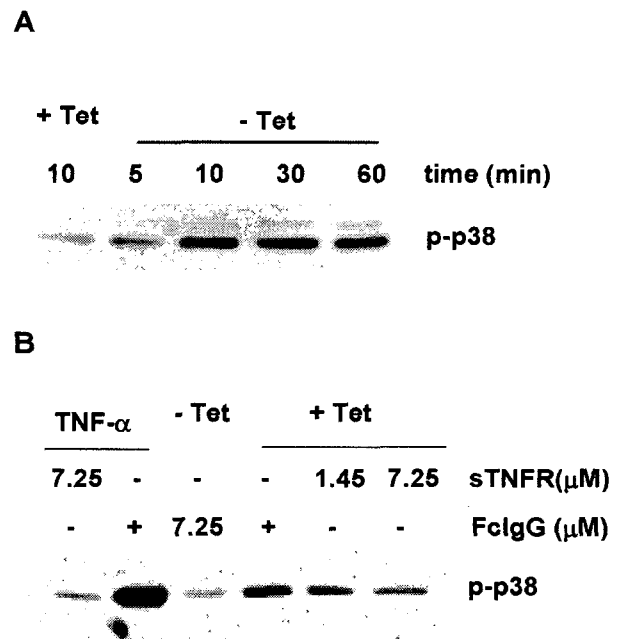


Fig. 2. Phosphorylation of p38 MAPK. (A) Normal rat SMCs were incubated for indicated period of time with the medium isolated from FADD-SMC grown in the absence (-Tet) or presence (+Tet) of tetracycline. Phosphorylated p38 MAPK in the lysate of the SMC was blotted with appropriate antibody. (B) Normal rat SMCs were incubated for 30 minutes with the culture medium isolated in the absence (-Tet) or presence (+Tet) of tetracycline with either sTNFR or control IgG (FcIgG). Then phosphorylated p38 MAPK was detected.

TNF- α to the increase of promoter activity, TNF- α in the medium was either neutralized with soluble TNF receptor (sTNFR) or incubated corresponding Fc portion of IgG (FcIgG), followed by evaluation of luciferase activity. The increased promoter activity was significantly blocked by sTNFR. These results indicate that the TNF- α in the medium play a role in phosphorylation of p38 MAPK and up-regulation of NF- κ B.

Cytotoxicity of the the secreted TNF- α

To examine cytotoxic activity of TNF- α in the culture medium collected from FADD-SMC in the absence of tetracycline, the culture medium was applied to L929 cells, which are killed by TNF- α at low concentrations. After incubation, DNA fragmentation was examined by ELISA (Fig. 4). The culture medium collected in the absence of tetracycline increased oligonucleosomes of normal SMC ($P < 0.01$), indicating apoptotic activity of the medium col-

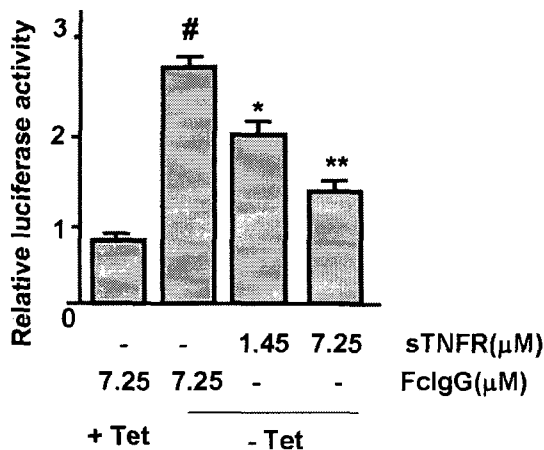


Fig. 3. Evaluation of luciferase activity. Rat SMCs were transfected with pNF-κB Luc plasmid using a lipofection reagent, and incubated for 20 hours with the medium isolated from FADD-SMC in the absence (-Tet) or presence (+Tet) of tetracycline. Luciferase activity in the SMC was examined. The normalized luciferase activity was reported as relative activity compared to that of control. Values are mean ± SE (n=4). #P<0.01 vs. -Tet; *P<0.05 vs. -Tet with FcIgG; **P<0.01 vs. -Tet with FcIgG.

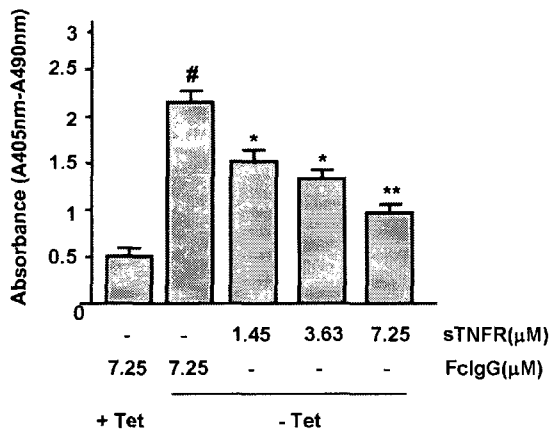


Fig. 4. Cytotoxicity of the medium collected from FADD-SMC. L929 cells were incubated with the medium collected from FADD-SMC in the absence (-Tet) or presence (+Tet) of tetracycline with or without sTNFR. Then, nucleosome formation was determined. Data are the representative of three independent experiments in triplicate. Data are presented as mean ± SD. #P<0.01 vs. -Tet; *P<0.05 vs. -Tet with FcIgG; **P<0.01 vs. -Tet with FcIgG.

lected from FADD-SMC in the absence of tetracycline. To assess role of TNF-α in the medium to the death activity, TNF-α in the medium was neutralized with soluble TNF receptor (sTNFR) and then death activity was determined.

The presence of sTNFR blocked oligonucleosome formation by the culture medium in a concentration dependent fashion (P<0.01).

Discussion

The present study demonstrated that SMC dying by FADD secreted TNF-α into culture medium. The secreted TNF-α participated in the phosphorylation of p38 MAPK and the up-regulation of NF-κB, and induced cytotoxicity of L929 cells.

The relationship between SMC death and early pathological changes in vascular diseases is still not clear partly due to limited availability of clinical specimens. Thus, it would be interesting to speculate biological consequences of SMC death. In the review of the role of apoptosis in atherosclerosis, Kockx and Herman pointed out that some aspects/consequences of apoptosis would be predicted to have clinical consequences that are beneficial, and others that are deleterious[15]. Apoptosis is a major mechanism by which tissues remove damaged and aged cells and is also widely recognized as a clean death, because apoptotic cells and bodies are recognized by adjacent professional and nonprofessional phagocytes and rapidly removed from the tissue[11]. Contrary to the generally held opinion that apoptosis is silent and non-inflammatory, there are reports that Fas-mediated apoptosis can trigger inflammatory reactions[4,20]. Furthermore, at late stage of apoptosis, cells are prone to undergo secondary necrosis and this would lead to inflammation. The results in this study suggest that induction of apoptosis SMC may result in up-regulation of pro-inflammatory genes. This up-regulation may normally serve a beneficial function in recruiting professional scavengers, especially macrophages, that help remove the cell and matrix debris that these structural cells leave behind. It is believed that apoptosis in vessels is related with lack of cellularity in vascular diseases[2,3,14]. Thus, death of SMC in vasculature may have some positive consequences, but massive death of SMC would leads to inflammation in vasculature and plaque instability.

TNF-α, a cytokine produced by many cell types including macrophages, monocytes, lymphocytes, and fibroblasts, in response to inflammation, infection, injury and other environmental challenges[12,17], amplifies inflammatory reactions, induce expression of MMPs and apoptosis of vascular cells that are known to contribute to atherogenesis. It

is hard to explain how up-regulation of TNF- α occurred in FADD-SMC as very little is known about transcriptional activation in response to death-pathway activation. It is generally believed that Fas-mediated pathway is a killer in that its activation of caspase and the resulting apoptosis require no transcriptional or translational component[17]. Thus, the mechanisms by which up-regulation of TNF- α occur in dying cells needs further investigations.

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초록 : FADD 과발현 평활근세포에서 분비하는 Tumor Necrosis Factor- α 의 작용

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세포 배양액에 tetracycline이 없는 경우 FADD를 발현하면서 사멸하는 평활근세포 (FADD-SMC)에서 분비하는 TNF- α 의 활성을 조사하였다. 배양액에 tetracycline이 없는 경우 FADD-SMC는 약 1000 pg/ml의 TNF- α 를 분비하였다. TNF- α 를 포함하는 배양액을 분리하고, 이 배양액을 정상세포에 처리한 결과 인산화한 p38 MAPK와 nuclear factor kappa B (NF- κ B)의 활성이 증가하였다. 또한 이 배양액을 L929 세포에 처리하는 경우 세포독성이 발생하였다. NF- κ B, p38 MAPK 그리고 L929 세포에 대한 효과는 배양액에서 soluble TNF receptor를 이용하여 TNF- α 를 제거하는 경우 감소하였다.