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Conditioned Medium from Dying Smooth Muscle Cell Induced Apoptotic Death

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In this study, the authors investigated whether death of vascular smooth muscle cell (VSMC) had a pathological pertinence. Conditioned media obtained from rat aorta smooth muscle cell (SMC) that were induced death by expressing FADD in the absence of tetracycline (FADD-SMC) triggered death of normal SMC. DNA fragmentation and caspase-3 activation were observed in dying SMC by conditioned media. FADD-SMC showed transcriptional activation of tumor necrosis factor (TNF)-α. Conditioned medium contained TNF-α, indicating secretion of the cytokine from dying FADD-SMC. It was investigated if secreted TNF-α was functional. Conditioned medium activated ERK and p38 MAPK pathways and induced MMP-9 expression, whereas depletion of the cytokine with its soluble receptor (sTNFR) remarkably inhibited induction of MMP-9 by conditioned medium. These findings suggest that TNF-α in conditioned medium seems to be active. Then, contribution of TNF-α on death-inducing activity of conditioned medium was examined. Depletion of TNF-α with soluble TNF-α receptor decreased the death activity of conditioned medium by 35%, suggesting that TNF-α play a partial role in the death activity. Boiling of medium almost completely abolished the death-inducing activity, suggesting that other heat labile death inducing proteins existed in conditioned medium. Taken together, these results indicate that SMC undergoing death could contribute to inflammation by expressing inflammatory cytokines and pathological complications by inducing death of neighboring cells.

Key Words: Apoptosis, FADD, TNF-α, Vascular smooth muscle

INTRODUCTION

Apoptosis is a type of genetically programmed cell death and a major mechanism by which tissues remove damaged and aged cells. Although cells in mammalian tissues have diverse phenotypes and genotypes, during the development of apoptosis, all cell types undergo similar morphological alterations (Vaux & Korsmeyer, 1999). Death of vascular smooth muscle cell (VSMC) is observed in vessel wall not only in physiological arterial remodeling (Cho et al, 1995) but also in disease states including atherosclerosis (Crisby et al, 1997; Kockx, 1998; Libby, 2001). In contrast to the notion that apoptotic cell death, a type of VSMC death in vasculature, is crucial in development and homeostasis in tissues for removal of either infected or damaged cells (Vaux & Korsmeyer, 1999), cell death including apoptosis in VSMC is believed to be deleterious in vascular diseases. Death of VSMC has been proposed to contribute to plaque hypocellularity (Bauriedel et al, 1999), blood coagulation (Flynn et al, 1997), and vascular calcification (Proudfoot et al, 2000). VSMC death also is believed to weaken texture and reduce deposition of extracellular matrix protein in the plaque, leading to plaque instability and rupture (Kockx & Herman, 1998; Bauriedel et al, 1999; Bennett, 1999). Plaque rupture and subsequently occurring thrombosis are crucial events in acute coronary syndromes and in the progression of underlying atherosclerotic diseases (Bauriedel et al, 1999; Kockx & Herman, 2000). Thus, better understandings of the mechanisms and the factors associated with VSMC death are of importance in therapeutic strategies for vascular diseases.

Among receptor-mediated death pathways, tumor necrosis factor (TNF)-α receptor (TNFR) and Fas/Fas ligand (FasL) pathway were demonstrated to play roles in VSMC death in the atherosclerotic lesions (Cai et al, 1997). Upon binding of ligands, members of the TNFR superfamily can induce cell death. The most widely accepted apoptotic death pathway involves TNFR1-associated death domain protein (TRADD), Fas-associated death domain-containing protein (FADD), and caspases. Binding of TNF-α to its receptor, TNFR1 induces receptor trimerization and recruitment of several signaling proteins to the cytoplasmic domains of the receptors. Among the recruited proteins, TRADD, FADD, and caspase-8 are associated with cell death (Baker & Reddy, 1998; Idriss & Naismith, 2000; MacEwan, 2002; Aggarwal, 2003). TNF-α alone does not induce VSMC death, since it also activates NF-κB-mediated cell survival

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ABBREVIATIONS: VSMC, vascular smooth muscle cell; SMC, smooth muscle cell; FADD, Fas-associated death domain-containing protein.

pathway (Van Antwerp et al, 1996). However, it was reported that TNF-α was present in the atherosclerotic plaques and would induce VSMC death with other cytokines (Geng et al, 1996).

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 (Chinnaiyan & Dixit, 1996) and is also involved in death induction by other death receptors of TNFR1 and DR3 (Baker & Reddy, 1998; Aggarwal, 2003). 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 overexpression of FADD triggered apoptotic death in cells (Chinnaiyan et al, 1995; Chinnaiyan et al, 1996). 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 (Walsh et al, 1998). FADD knockout mouse is embryonic lethal. This suggests that FADD is required for embryonic development (Yeh et al, 1998).

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 (Schaub et al, 2000). Fas stimulation triggered neo-angiogenesis and local infiltration of inflammatory cells, independently of apoptosis (Biancone et al, 1997). However, the pathological effect of Fas-mediated apoptosis to neighboring cells is unknown. The aim of this study was to investigate any pathological role of apoptotic death. To investigate the effect of apoptotic death to neighboring cells *in vitro*, death-inducing molecule should be removed from conditioned medium to be used, which is practically very difficult. Thus, experiments were performed using smooth muscle cells that undergo death by overexpressing FADD.

METHODS

Cell culture

The aorta smooth muscle cells from Fisher rat were grown in Dulbecco's modified Eagle's medium-high glucose (DMEM) (Life Technology, Grand Island, NY) supplemented with 10% calf serum, 5 mM L-glutamine, plus 50 units/ml penicillin and 50 μg/ml streptomycin in a humidified atmosphere of 5% CO_2.

Antibodies and reagents

Antibodies for activated caspase-3, phospo-ERK and phospho-p38 MAPK were from New England Biolabs Inc. (Beverly, MA). Anti-FADD antibody was from BD Transduction Laboratories (San Diego, CA). The rabbit polyclonal antibody raised against MMP-9 was kindly provided by Dr. Moon (Institute of Biotechnology, Euven, Belgium). Anti-alpha-tubulin antibody was from Oncogene Research Products (San Diego, CA). Anti-rat TNF-α antibody was purchased from R&D systems (Minneapolis, MN). The soluble TNF receptor (sTNFR) was a generous gift from Immunex Corporation (Seattle, WA).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from SMC using the TRIZOL reagent (Life Technologies) following the manufacturer's instructions. One microgram of the isolated total RNA was reverse-trascribed into cDNA with the Superscript™ Pre-amplification System (Life Technologies). After reverse transcription reaction, the reaction was diluted with double distilled water (1 : 1) and incubated at 94°C for 10 mins. The primers for rat TNF-α were 5'-CTCTTCTCATTCCC GCT CGTG-3' and 5'-ATGGCGGAGAGGAGGCTGACT-3', yielding a 401 bp product. The primers for glycerol aldehyde 3-phosphate dehydrogenase (GAPDH) were 5'-TTGTT GCCATCAATGACCC-3' and 5'-CATGAGTCCTTCCACGAT ACC-3'. The reaction of PCR was composed of 50 mM KCl, 10 mM TrisHCl (pH 9.0 at 25°C), 10 μM of each primers, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 1.25 U of Taq polymerase (Promega, Madison, WI), and 5 μl of diluted reverse transcription reaction. The cDNA was amplified in a GeneAmp PCR System 9600 (PerkinElmer Inc., Wellesley, MA) by 30 cycles of PCR (94°C for 30 secs, 55°C for 40 secs, and 72°C for 45 secs). The product was separated on agarose gels containing 0.5 μg/ml ethidium bromide and photographed.

Zymography

Conditioned media were subjected to zymography according to the procedure previously described (Herron et al, 1986). Equal volumes of conditioned media were loaded onto 8% polyacrylamide gels containing 0.1% of type I gelatin (Sigma Chemical Co., St. Louis, MO) and electrophoresed at a constant voltage. After electrophoresis, gels were rinsed in 2.5% Triton X-100 for 30 mins, incubated for 16 to 18 hrs at 37°C in a buffer (50 mM TrisHCl, pH 8.0, 2.5 mM $CaCl_2$, and 0.02% sodium azide), rinsed in 10% trichloroacetic acid, and stained in rapid Coomassie stain. The stained gels were visualized by Eagle-Eye Image (Stratagene, La Jolla, CA).

DNA laddering

Adherent and floating cells were lysed in DNA extraction buffer (20 mM TrisHCl pH 7.4, 100 mM NaCl, 5 mM EDTA pH 8, 0.5% SDS). The cell lysate was incubated with 200 μg/ml proteinase K overnight at 37°C. Nucleic acid was extracted with an equal volume of phenol-chloroform (1 : 1). After mixing by gentle repetitive pipeting, the mixture was centrifuged at 14,000 xg for 20 mins. Aqueous phase containing nucleic acid was transferred to a new tube. RNA was digested by incubating with 5 μg/ml DNase free RNase for 2 hrs at 37°C. DNA was extracted with phenol- chloroform again and precipitated overnight at −20°C. After centrifugation at 14,000 xg for 20 mins at 4°C, DNA pellet was washed with 70% ethanol. The pellet was dissolved in 10 mM TrisHCl and 1 mM EDTA solution (pH 7.4) and stored in the refrigerator overnight. The concentration of DNA was determined by spectrophotometry. The purified DNA was separated on agarose gels containing ethidium bromide and visualized under UV.

Measurement of oligonucleosome

Oligonucleosomes in cells treated with appropriate reagents were quantitatively determined with the cell-death detection ELISA^{PLUS} kit following manufacturer's instructions (Roche, Indianapolis, IN). Cells were collected, resuspended in the lysis buffer provided in the kit, and incubated for 30 mins at room temperature. The resultant supernatants after centrifugation at 200 g for 10 mins were transferred into the streptavidin coated microplate with the immunogen reagent. After incubation for 2 hrs 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.

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 the BCA Protein Assay Reagent (Bio-Rad, Hercucles, CA). Twenty micrograms of protein were separated on SDS-PAGE gels and transferred to polyvinylidine difluoride membrane. Nonspecific binding sites were blocked in T-TBS (50 mM TrisHCl, pH 7,4, 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk for 2 hrs at room temperature. The membrane was incubated with primary antibodies in T-TBS at 4°C overnight. After washing with T-TBS, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody. Being washed three times with T-TBS, membrane was incubated with Enhanced Luminol Reagent (NEN, Boston, MA). The chemiluminescent signal was imaged on the X-ray film.

RESULTS

Controlled regulation of expression of FADD and cell death in FADD-SMC

The present study used conditioned medium of SMC undergoing death by overexpression of FADD in the absence of tetracycline (FADD-SMC) (Schaub et al, 2000). Thus, a tight regulation of expression of FADD and death by FADD was important. Regulation of FADD expression by tetracycline was examined (Fig. 1). Cells were cultured in the absence or presence of tetracycline for 1, 2, and 3 days and FADD was detected by Western blot analysis. Immunoreactivity of FADD was observed at day 1 after removal of tetracycline. The immunoreactivity increased in a time dependent manner. FADD was not detected from cells cultured in the presence of tetracycline. Viability was determined by trypan blue exclusion after cells were cultured for 1, 2 and 3 days in the absence and presence of tetracycline. Cell death was observed from SMC cultured in the absence of tetracycline in a time dependent fashion. The death, however, did not occur in the presence of tetracycline. The results indicate that expression of FADD and death in FADD-SMC are tightly regulated by tetracycline.

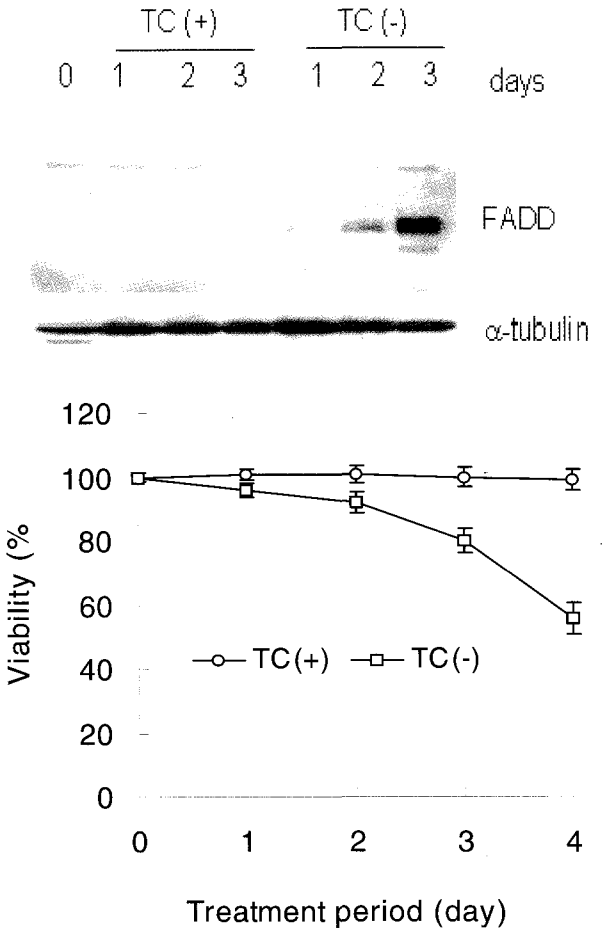

Fig. 1. Tight regulation of FADD expression and death induction in FADD-SMC by tetracycline. FADD-SMCs were cultured in the absence [TC (−)] or presence [TC (+)] of tetracycline. Cells were lysed at the indicated time point. The lysates were subjected to Western blot analysis using antibody against FADD (the upper panel). Viability was determined by trypan blue exclusion at the indicated period of time (the lower panel).

Conditioned medium from FADD-SMC induced cell death

To investigate any pathological consequence of cell death, conditioned medium of FADD-SMC was prepared with or without tetracycline and applied to aorta SMC from Fisher rat (normal SMC). Morphological appearance of normal SMC treated with indicated conditioned medium was examined (Fig. 2). SMC treated with conditioned medium isolated with tetracycline remained attached to tissue culture plates. However, SMCs treated with conditioned medium isolated without tetracycline lost adherence and were floating in the medium. To rule out any effect by tetracycline, an equal amount of tetracycline was added just prior to use.

It was investigated if the death of normal SMC by conditioned medium showed apoptotic features. As DNA fragmentation is one of the features in apoptotic death, the authors examined occurrence of apoptosis by detecting fragmentation of chromosomal DNA (Fig. 3A). A typical

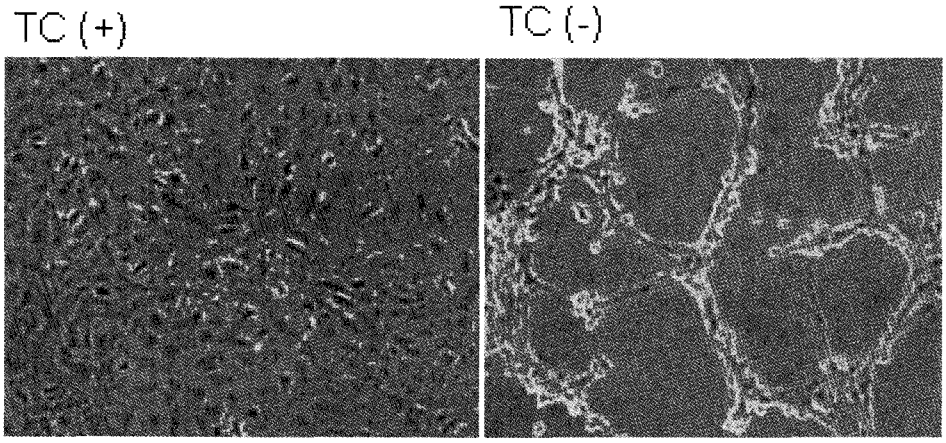

Fig. 2. Morphology of Fisher rat aorta SMC (normal SMC) incubated with conditioned medium of FADD-SMC. Fisher rat aorta SMCs were exposed to conditioned medium from FADD-SMC cultured either with [TC (+)] or without [TC (−)] tetracycline. Cells were visualized and photographed at 72 hrs post exposure.

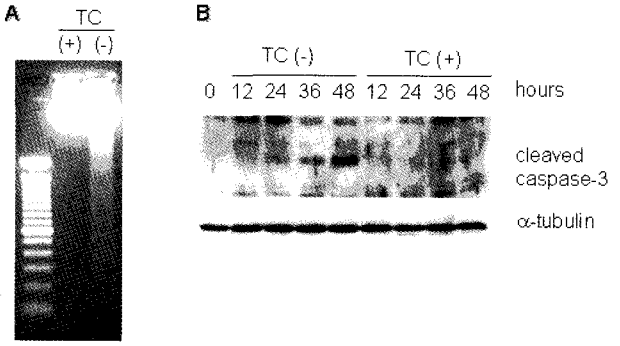

Fig. 3. Apoptotic features in Fisher rat aorta SMC undergoing death by conditioned medium of FADD-SMC. (A) DNA fragmentation in SMC treated with conditioned medium prepared without tetracycline. Genomic DNA isolated from SMC by phenol- chloroform extraction after treatment with conditioned medium prepared with [TC (+)] or without [TC (−)] tetracycline was electrophoresed on a 2% agarose gel and stained with ethidium bromide. The first lane shows size markers of the 100 bp ladder. (B) Lysates prepared from SMC treated for indicated period with conditioned medium prepared with [TC (+)] or without [TC (−)] tetracycline were subjected to Western blot analysis with antibody that recognizes active form of caspase-3.

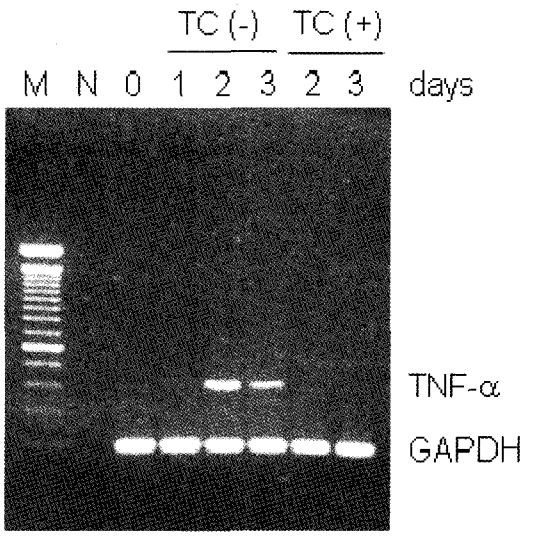

Fig. 4. Transcriptional activation of TNF-α in FADD-SMC in the absence of tetracycline. Total RNA was isolated from FADD-SMC cultured for the indicated period in the absence [TC (−)] or presence [TC (+)] of tetracycline. TNF-α transcript in the isolated RNA was amplified by RT-PCR. Lane N is the control (no cDNA). Lane M shows the 100 bp DNA marker. The position of TNF-α and GAPDH product is indicated.

electrophoretic pattern of oligonucleosomal DNA laddering was observed by conditioned medium isolated without tetracycline (lane 2). The degradation of chromosomal DNA requires activation of caspase-3. Thus, active (cleaved) form of caspase-3 was detected by Western blot analysis after treatment with conditioned medium (Fig. 3B). The cleaved caspase-3 was detected at 24 hrs after treatment with conditioned medium of FADD-SMC prepared without tetracycline and persisted thereafter. The results indicate that conditioned medium from the cells undergoing death by FADD induces apoptotic death in normal SMC.

Expression of cytokine from FADD-SMC

The induction of apoptotic death by conditioned medium of FADD-SMC indicated existence of soluble apoptosis-inducing molecules in the medium. As cytokines, including TNF-α, were reported to induce SMC death, it was investigated whether FADD-SMC expressed and secreted the cytokines. FADD-SMCs were cultured in the absence and presence of tetracycline and expression of TNF-α was examined by RT-PCR (Fig. 4). Whereas transcript of TNF-α was not detected from FADD-SMC that did not undergo apoptotic death in the presence of tetracycline, it was detected from FADD-SMC cultured in the absence of tetracycline. The transcript appeared at day 1 after removal

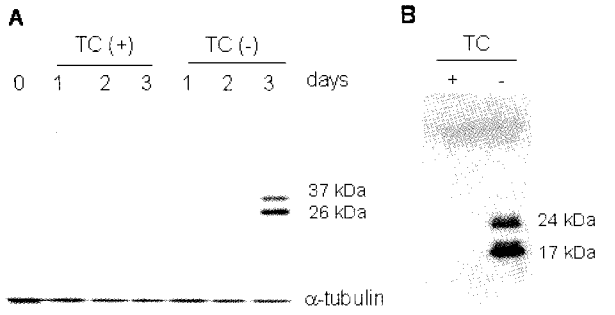

Fig. 5. Expression and secretion of TNF-α by FADD-SMC in the absence of tetracycline. (A) FADD-SMCs were cultured for 0, 1, 2, and 3 days in the absence [TC (−)] or presence [TC (+)] of tetracycline. The lysates were prepared and subjected to Western blot analysis using antibodies against rat TNF-α. (B) Conditioned medium was collected at day 3.5 from FADD-SMC cultured with (+) or without (−) tetracycline. The collected medium was concentrated with the Centricon-3. The concentrated medium was subjected to Western blot analysis for TNF-α.

of tetracycline and persisted thereafter.

The presence of transcript does not necessary mean that protein is synthesized. Thus, translation of TNF-α by FADD-SMC was examined (Fig. 5A). FADD-SMCs were cultured in the absence or presence of tetracycline for indicated periods and TNF-α in cell lysates was detected by Western blot analysis. Two bands of TNF-α immunoreactivity were detected from FADD-SMC cultured in the absence of tetracycline. The lower band represents TNF-α with a molecular weight of 26-kDa. The upper band is likely to be a precursor form of TNF-α. No TNF-α immunoreactivity, however, was observed in FADD-SMC cultured in the presence of tetracycline.

In order to be secreted, TNF-α should be cleaved to a 17-kDa soluble form and the secreted 17-kDa TNF-α exerts biological effects (Idriss & Naismith, 2000). Thus, secretion of TNF-α by FADD-SMC was investigated in the absence or presence of tetracycline (Fig. 5B). Immunoreactive TNF-α was detected only from conditioned medium prepared without tetracycline. TNF-α immunoreactivities with molecular wight of 17 kDa and 24 kDa were the major form in conditioned medium collected without tetracycline.

Secretion of active TNF-α by FADD-SMC

Activity of TNF-α in conditioned medium was examined. As TNF-α induces expression of MMP-9 via activation of p38 and ERK in rat SMC used in this study (Cho et al, 2000), it was investigated if conditioned medium isolated without tetracycline activated p38 and ERK and induced expression of MMP-9 (Fig. 6). Treatment with conditioned medium isolated without tetracycline elevated the levels of phosphorylated p38 and ERK, whereas the elevation was not observed by treatment with conditioned medium isolated with tetracycline. The results suggest activation of p38 and ERK pathways by conditioned medium collected without tetracycline.

It was investigated whether expression of MMP-9 was influenced by conditioned medium. When SMCs were treated with conditioned medium isolated without tetracycline, activity and immunoreactivity of MMP-9 were remarkably increased (Fig. 7A). The increase, however, did not occur

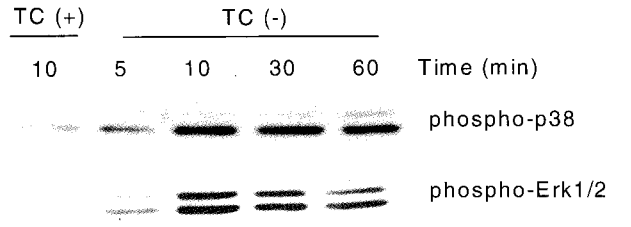

Fig. 6. Activation of MAPKs by conditioned medium from FADD-SMC. Fisher rat SMCs were treated for indicated period with conditioned medium isolated with [TC (+)] or without [TC (−)] tetracycline. Treated cells were lysed and lysates were subjected to Western blot analysis using antibodies against phospho-p38 MAPK and phospho-ERK.

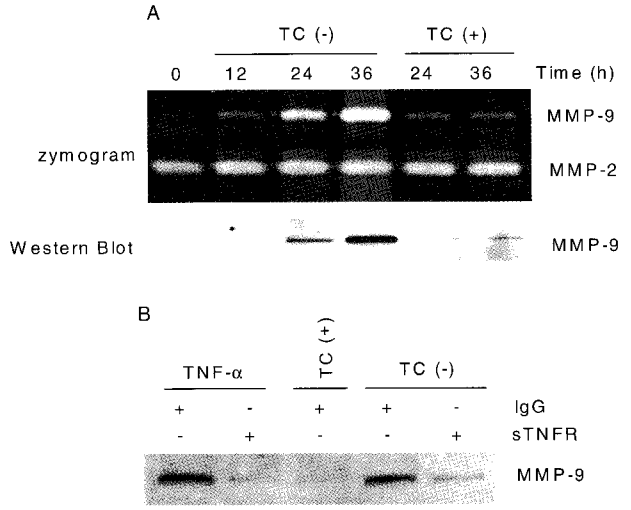

Fig. 7. Up-regulation of MMP-9 by conditioned medium of FADD-SMC. (A) Fisher rat SMCs were treated for indicated period with conditioned medium isolated with [TC (+)] or without [TC (−)] tetracycline. The activity of MMP-9 was investigated with the lysates of the SMC. The identical samples were subjected to Western blot analysis using antibodies against MMP-9. (B) Fisher rat SMCs were treated with indicated reagents. Lysates prepared with the treated cells were subjected to Western blot analysis using antibodies against MMP-9.

by conditioned medium prepared with tetracycline. Next, effect of depletion of TNF-α on expression of MMP-9 was investigated after TNF-α in conditioned medium had been depleted with soluble tumor necrosis factor-α receptor (sTNFR). The sTNFR efficiently inhibited expression of MMP9 by TNF-α. Incubation of conditioned medium with sTNFR remarkable inhibited expression of MMP-9 induced in SMC by conditioned medium isolated without tetracycline. The results indicate that conditioned medium isolated in the absence of tetracycline contains active TNF-α that is able to activate p38 and ERK and induce expression of MMP-9.

Partial inhibition of SMC death by TNF-α depletion

As sTNFR efficiently inhibited activity of TNF-α, it was investigate if an identical concentration of sTNFR affected death of normal Fisher cells by conditioned medium

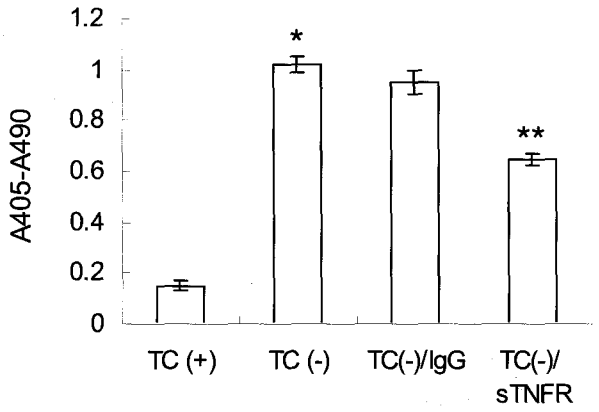

Fig. 8. Inhibition of lethal effect of conditioned medium from FADD-SMC by sTNFR. Fisher rat aorta SMCs were exposed for 72 hrs to conditioned medium isolated from FADD-SMC cultured either with [TC (+)] or without [TC (−)] tetracycline in the absence or presence of sTNFR or control IgG. Oligonucleosomes in the cells were measured as described in material and method. Data are expressed as mean±SD (n=4). Differences between groups were analyzed by Students' *t*-test. *P<0.01 vs. TC (+), **P<0.05 vs. TC (−).

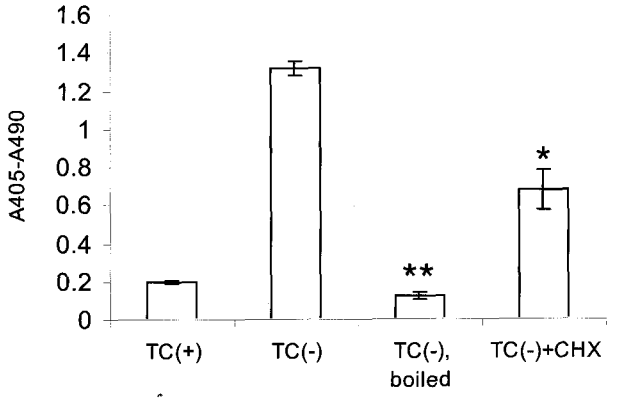

Fig. 9. Viability of SMC treated with conditioned medium from FADD-SMC. Fisher rat aorta SMCs were exposed for 72 hrs to boiled conditioned medium isolated without tetracycline [TC (−)] or to conditioned medium isolated from FADD-SMC cultured with [TC (+)] or without [TC (−)] tetracycline in the absence or presence of cycloheximide (CHX). Oligonucleosomes in the cells were measured. Data are expressed as mean±SD (n=4). Differences between groups were analyzed by Students' *t*-test. **P<0.01 vs. TC (−), *P<0.05 vs. TC (−).

isolated without tetracycline (Fig. 8). The depletion of TNF-α with sTNFR decreased the lethal effect of conditioned medium by 35% ($P<0.05$), suggesting TNF-α played a partial role in the SMC death. Additional experiments were performed to explore what kind of molecules in conditioned medium caused SMC death (Fig. 9). Conditioned medium was boiled prior to addition. Boiling almost completely abolished death activity of conditioned medium ($P<0.01$), indicating death inducing molecule is heat-labile. When conditioned medium was co-treated with a protein synthesis inhibitor, cycloheximide, cell death was also significantly inhibited ($P<0.05$). The results suggest that protein synthesis is necessary to exert full activity of death induction.

DISCUSSION

The present study demonstrated transcriptional activation and translation of TNF-α in dying SMC by overexpression of FADD. TNF-α is a cytokine produced by many cell types including macrophages, monocytes, lymphocytes, and fibroblasts, in response to inflammation, infection, injury and other environmental challenges (Idriss & Naismith, 2000; MacEwan, 2002). The cytokine can amplify inflammatory reactions, induce expression of cell adhesion molecules, and apoptosis of vascular cells that are known to contribute to atherogenesis. It is difficult to explain how transcription and translation of TNF-α occurred in SMC by FADD 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 component (Nagata, 1999).

The ability of TNF-α to elicit dual responses of proliferation and cell death (Van Antwerp et al, 1996; MacEwan, 2002) raised the question as to whether other members of TNF family, like Fas, can do the same. This is likely to happen because it has been reported that in some situations and/or cell types Fas pathway is associated with proliferation instead of death. Thus, it may be possible that other signaling pathways might be activated during prolonged apoptosis.

What are the biological consequences of VSMC death? The relationship between SMC death and pathological change in vascular diseases is still not clear. However, it is believed that apoptosis in vessels is related with lack of cellularity in vascular diseases (Libby et al, 1997; Kockx & Herman, 1998; Bauriedel et al, 1999; Bennett, 1999). Apoptosis is 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 (Henson et al, 2001). Unfortunately, apoptosis is not the only type of cell death in vascular diseases. A number of necrotic cells were detected in atherosclerosis (Crisby et al, 1997). The necrosis in part might result from inefficient clearance of apoptotic cells. This would contribute to pathological changes in vascular diseases. At late stage of apoptosis, cells are prone to undergo secondary necrosis and this would lead to inflammation. Unlike necrosis, apoptosis is classically considered to be "silent" (i.e., non-inflammatory). This notion is based largely on findings in leukocytes (Nagata, 1999; Vaux & Korsmeyer, 1999).

The results in this study suggest that induction of apoptosis in cell types in which the cell body and extracellular matrix play structural/mechanical roles (e.g., 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. In their 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 (Kockx & Her-

man, 2000). It is possible that the pro-inflammatory program of SMC apoptosis may have significant adverse clinical consequences at sites of massive apoptosis (e.g., after myocardial infarction), and that in these cases, it would be beneficial to partially inhibit that program. To do this, the signaling pathways leading from Fas to gene expression need to be identified so that specific inhibitors can be developed.

The ERK and the p38 MAPK pathways were shown to regulate MMP-9 expression. For example, overexpression of dominant-negative MEK1 inhibited the MMP-9 expression in PMA-treated carcinoma cells (Gum et al, 1997). In a separate study, p38 MAPK was involved in PMA-induced MMP-9 secretion with use of the SB203580 compound (Simon et al, 1998). The contribution of both pathways to the transcriptional regulation of MMP-9 in arterial smooth muscle was reported (Cho et al, 2000). In the present study, we demonstrated that the TNF-α in conditioned medium induced expression of MMP-9 in SMC and that this induction was related with up-regulation of p38 MAPK and ERK. It would be of interest to speculate whether induction of MMP-9 expression has physiological meanings? Degradation of extracellular matrix by matrix metalloproteinases (MMPs) is thought to be important in the progression of atherosclerosis and plaque rupture. MMP-1, MMP-3, and MMP-9 were identified in human atherosclerotic lesions, and the enhanced expression of MMP-9 at the shoulders of these lesions was linked to plaque rupture (Galis et al, 1994; Wesley et al, 1998). In experimental animal models, MMPs were also shown to be important for SMC migration into the intima. In a rat arterial injury model, after the initial medial SMC replication, medial SMC migrate and first appear in the intima 4 days after injury. MMP-9 was expressed within 6 hrs after injury in rat carotid arteries and continues to be expressed up to 6 days (Clowes et al, 1983; Zempo et al, 1994; Cho & Reidy, 2002). The importance of these MMPs in the migration of SMC is illustrated by the finding that the administration of MMP inhibitor almost completely inhibits the number of SMC migrating into the intima (Bendeck et al, 1994). These findings suggested that MMP-9 would be important in the lesion growth.

In summary, the present study demonstrate that SMC dying by expression of FADD secreted active TNF-α into medium. TNF-α in the medium activates MAPKs, upregulates MMP-9, and partially contributes to the death in the normal SMC. This study reports that VSMC death might be associated with inflammation and lesion progression.

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