Expression of Tumor Necrosis Factor (TNF)-α from Cells Undergoing Death by FADD

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Abstract Apoptosis of vascular smooth muscle cell is served in the vascular diseases such as atherosclerosis and stenosis. The death of vascular smooth muscle cells can be induced by cytokines and activation of Fas-pathways. It is widely accepted that apoptosis occurs without inflammation. There are, however, reports that apoptosis is not silent. Vascular smooth muscle cells dying by Fas-pathway secreted inflammatory cytokines including monocyte chemotactic protein-1. This study have investigated whether apoptosis is associated with potent inflammatory cytokine tumor necrosis factor (TNF)-α. The cells which undergo apoptosis by expressing FADD in the absence of tracycline expressed and secreted TNF-α. When the level of TNF-α transcript was investigated, dying smooth muscle cells exhibited transcriptional activation of TNF-α. The data indicate that dying vascular smooth muscle cells contribute to inflammation by expressing inflammatory cytokines. The present study suggests that apoptosis could not be silent in certain pathological situations.

Key words: Tumor necrosis factor, FADD, Apoptosis

Introduction

FADD was identified as an adaptor molecule linking the activated Fas (CD95) receptor to the effector molecule caspase-8 and is essential for apoptosis signaling of Fas receptor. FADD is also involved in apoptosis induction by other death receptors of TNFR1 and DR3. FADD contains a death domain (DD) at its C-terminus. The DD domain binds to 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 apoptosis signaling as overexpression of FADD triggered apoptosis in cells [3,4]. 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[11]. FADD knockout mouse is embryonic lethal. This suggests that FADD is required for embryonic development[12].

Apoptosis is considered silent without inflammation. It is a type of genetically programmed cell death and a major mechanism by which tissue removes 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[14]. Contrary to generally held opinion that apoptosis is non-inflammatory, there are reports that Fas-mediated apoptosis can trigger inflammatory reactions. FADD-induced apoptosis resulted in a massive inflammatory response[10]. Fas stimulation triggered neo-angiogenesis and local infiltration of inflammatory cells, independently of apoptosis[2]. In the present study, it was investigated whether vascular smooth muscle cells (VSMCs) dying by FADD expressed a potent proinflammatory cytokine TNF-α. This study reports that TNF-α is secreted from cells undergoing death by FADD.

Materials and Methods

Cell Culture

Rat smooth muscle 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 μg/ml streptomycin in a humidified atmosphere of 5% CO2.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from smooth muscle cells using
TRIZOL reagent (Life Technologies) following manufacturer’s instructions. One microgram of total RNA was reverse-transcribed into cDNA with Superscript™ Preamplification System (Life Technologies). After the reverse transcription reaction, the reaction was diluted with double distilled water (1:1) and incubated at 94°C for 10 min. The primers for rat TNF-α were 5'-CTTTCTTCATTCCCCGCCTGGT-3' and 5'-ATGGCGGAGGAGGCTGACT-3', yielding a 401-bp product. The reaction of PCR was composed of 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 10 μM of each primers, 1.5 mM MgCl₂, 0.2 mM of each dNTPs, 1.25 U of Taq polymerase (Promega), and 5μl of diluted reverse transcription reaction. cDNA was amplified in a GeneAmp PCR System 9600 (Perkin Elmer) by 30 cycles of PCR (94°C for 30 sec, 55°C for 40 sec, and 72°C for 45 sec). The product was separated on agarose gels containing 0.5 μg/ml ethidium bromide and photographed.

### Western blot analysis

Cells were lysed in a lysis buffer (50 mM TrisCl, pH 7.8, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.1 mM phenylmethylsulfonyl fluoride). The protein content was determined using BCA Protein Assay Reagent (Pierce). Twenty micrograms of protein was separated on SDS-PAGE gels and transferred to polyvinylidene difluoride membrane (Millipore Co.). 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 times washing with T-TBST, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody. Being washed 3 – 4 times with T-TBST, the membrane was incubated with Enhanced Luminol Reagent (NEN). The chemiluminescent signal was imaged on the X-ray film.

### Results

#### Expression of FADD in the absence of tetracycline

To investigate relationship between apoptosis and TNF-α, the cell line that dies by expression of FADD in the absence of tetracycline was utilized [10]. The regulation of FADD expression by tetracycline was examined (Fig. 1). Cells were cultured with or without tetracycline for 1, 2, and 3 days. FADD in the cell was detected by Western blot analysis. Immunoreactivity of FADD was observed at day 1 after removal of tetracycline. The immunoreactivity was increased in a time dependent manner. But no FADD was detected from cells cultured in the presence of tetracycline. The data suggest that expression of FADD is tightly regulated.

#### Expression of TNF-α from cells dying by FADD

It was investigated whether VSMCs destined to die expressed TNF-α with FADD-expressing cells. Cells were cultured in the absence and presence of tetracycline. The expression of TNF-α was examined by RT-PCR (Fig. 2A). Transcript of TNF-α was not detected from cells that do not undergo apoptosis in the presence of tetracycline. The TNF-α transcript, however, was detected in cells cultured without tetracycline. The transcript was appeared at day 1 after removal of tetracycline and persisted thereafter. The presence of transcript does not necessary mean that protein is synthesized. Thus, the translation of TNF-α in the FADD-expressing cells was determined (Fig. 2B). Cells were cultured with or without tetracycline for indicated periods. TNF-α in the cell lysate was detected by Western blot analysis. Two bands of TNF-α immunoreactivity were detected from cells cultured without tetracycline. Lower bands represent 26 kDa TNF-α. Upper band is likely to be the precursor form of TNF-α. No TNF-α immunoreactivity, however, was observed in FADD-expressing cells cultured in the presence of tetracycline.

#### Secretion of TNF-α by FADD-expressing cells

TNF-α is can be cleaved to a 17 kDa soluble form and secreted [7]. The secreted 17 kDa TNF-α exerts biological effects. To investigate whether TNF-α was secreted from dying cells, the presence of TNF-α in the conditioned medium was determined. TNF-α was detected only from the conditioned medium collected in the absence of tetracycline (Fig. 3A). Two types were the major form in the conditioned medium. To identify the type of secreted TNF-α, the sizes of TNF-α in the cell and conditioned medium were compared. The sizes of TNF-α in the conditioned medium were 17 kDa and 24 kDa (Fig. 3B).

#### Discussion

Tumor necrosis factor (TNF-α) is a cytokine produced by many cell types including macrophages, monocytes, lymphocytes, and fibroblasts, in response to inflammation,
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Fig. 2. Expression of TNF-α from dying cells.
(A) Visualization of PCR products. Total RNA was isolated from FADD-expressing cells cultured with (TC+) or without (TC-) tetracycline at the indicated time period. TNF-α transcript in the isolated RNA was amplified from by RT-PCR. Lane N is the control (without cDNA). The first lane shows 100 bp DNA marker (Life Technology). The both positions of TNF-α and actin were should on the gel. (B) Detection of TNF-α protein synthesized in FADD-expressing cells in the absence of tetracycline. Cells were cultured with (TC+) or without (TC-) tetracycline and lysed at day 0, 1, 2, and 3. The lysates were subject to Western blot analysis using antibodies against rat TNF-α (R & D Systems).

Fig. 3. Secretion of TNF-α from dying cell.
(A) Detection of TNF-α in the conditioned medium of FADD-expressing cells. Conditioned medium was collected at day 3.5 from FADD-expressing cells cultured with (lane 1) or without (lane 2) tetracycline. Being concentrated with Centricon-3, the concentrated medium was subjected to Western blot analysis for TNF-α. The position of TNF-α in the medium is indicated. (B) Types of secreted TNF-α. Immunoreactive TNF-α was detected from the FADD-expressing cells (lane 1) and the conditioned medium isolated from FADD-expressing cells cultured in the absence of tetracycline (lane 2).

happen because it has been reported that in some situations and/or cell types Fas pathway is associated with proliferation instead of death. It is possible that other signaling pathways might be activated during prolonged apoptosis.

What are the biological consequences of death in VSMCs? The relationship between SMC death and pathological change in vascular diseases is still not clear. It is believed that apoptosis in vessels is related with lack of cellularity in vascular diseases[1]. Apoptosis 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[6]. Apoptosis is not the only type of cell death in vascular diseases. A number of necrotic cells were detected in atherosclerosis[5]. The necrosis in part might result from inefficient clearance of apoptotic cells. This would contribute to pathological changes. At late stage of apoptosis, cells are prone to undergo secondary necrosis and this would lead to inflammation.

References


