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Recombinant TFPI-2 enhances macrophage apoptosis through upregulation of Fas/FasL

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ABSTRACT

Tissue factor pathway inhibitor-2 (TFPI-2) is a Kunitz-type serine proteinase inhibitor with inhibitory activity toward activated factor XI, plasma kallikrein, plasmin, certain matrix metalloproteinases, and the tissue factor-activated factor VII complex. In addition, TFPI-2 has other functions such as promoting cell migration and inducing apoptosis. In the present study, we investigated if TFPI-2 induced apoptosis in cultured U937-derived macrophages and the possible signal pathways that involved in the apoptotic process. Apoptotic DNA fragment detection and caspase-3,9 activity measurements indicated that rTFPI-2 promoted U937-derived macrophage apoptosis. Hoechst 33342 assay and flow cytometry further showed that rTFPI-2 induced apoptosis in cultured macrophages in a dose-dependent manner. Because death receptors of the TNF family such as Fas are the best-understood death pathways that recruit Fas-associated death domain (FADD) and procaspase-8 to the receptor in macrophages, we investigated the expression of Fas and its ligand (FasL) and downstream signal caspase-8 by Western blot analysis. The results indicated that the process of apoptosis triggered by rTFPI-2 was, at least in part, actively conducted by U937-derived macrophages possibly through Fas/FasL signal pathway. In brief, rTFPI-2 may have the potential usefulness in inducing macrophages apoptosis, which suggest TFPI-2 might have antiatherogenic effects.

1. Introduction

Tissue factor pathway inhibitor-2 (TFPI-2) is a Kunitz-type serine proteinase inhibitor (Butzow et al., 1988; Sprecher et al., 1994), mainly synthesized by endothelial cells, vascular smooth muscle cells and syncytiotrophoblasts (Iino et al., 1998; Udagawa et al., 1998). Recombinant TFPI-2 exhibits strong inhibitory activity toward trypsin, factor XIa, plasma kallikrein, and plasmin, and weaker inhibitory activity for tissue factor–factor VIIa complex, factor IXa-polylysine, and cathepsin G, but failed to significantly inhibit the amidolytic activities of glandular kallikrein, urinary plasminogen activator, tissue plasminogen activator, activated protein C, factor Xa, thrombin, and leukocyte elastase (Petersen et al., 1996; Kong et al., 2004). In one or more of these roles, TFPI-2 might have important regulatory function in healthy and/or atherosclerotic vessels.

Although the importance of TFPI-2 as an inhibitor of thrombin generation remains unclear, a recent report has implicated that the interaction of TFPI-2 with TF:FVIIa in promoting cell migration (Neaud et al., 2000), although the physiological relevance of this process is yet to be elucidated. A further action of TFPI-2 as a vascular smooth muscle cell (VSMC) mitogen has also been suggested (Shinoda et al., 1999). Furthermore, TFPI-2 may represent a mechanism for negative feedback regulation and modulation of its pro-angiogenic action on endothelial cells (Xu et al., 2006) and might be an important regulator of aberrant angiogenesis (Ivanciu et al., 2007), suggesting that TFPI-2 might be a potential therapeutic for angiogenic disease processes.

Apoptosis, the programmed cell death, is critical for the development and maintenance of healthy tissues. A recent report (George et al., 2007) has implicated TFPI-2 activated both intrinsic and extrinsic caspase-mediated, proapoptotic signaling pathways and induced apoptosis in U-251 cells (a human glioblastoma cell line). Atherosclerosis can be considered to represent an inflammatory response of macrophages to 'invading' pathogenic lipoproteins in the arterial wall (Glass and Witztum, 2001). A recent report described the apparent downregulation of TFPI-2 in macrophages of atherosclerotic tissues (Herman et al., 2001), which also suggested the loss of TFPI-2 might promote plaque formation and/or complication.

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Therefore, TFPI-2 may play an important role in the pathogenesis of atherosclerosis via induction of apoptosis of macrophages. We want to learn more about TFPI-2's role in atherosclerosis, to know whether TFPI-2 could promote macrophages apoptosis and the possible signal pathways involved in the apoptotic process.

2. Materials and methods

2.1. Cell culture

Human monocytic leukemic cell line U937 was obtained from the cell bank of the Shanghai Institute of Biological Sciences, the Chinese Academy of Sciences. Cells used in experiments were from 5 to 10 passages. U937 cells were cultured in RPMI 1640 (GIBCO) with 10% (v/v) fetal bovine serum (FBS) (GIBCO), 100 U/ml penicillin, 100 μg/ml streptomycin, at 37 °C in a 5% (v/v) CO_2 humidified incubator. After an initial incubation in medium plus 10% FBS, cells were counted and adjusted to 3×10^5 cells/ml, followed by transferring 2 ml to each well of a 6-well tissue-culture plate, 1 ml to each well of a 24-well plate, and 100 μl each well of a 96-well plate. In experiments U937 cells were differentiated to resident macrophage-like cells by addition of 100 nmol/ml Phorbol 12-myristate 13-acetate (PMA) (Sigma) without changing the medium for 72 h. Cells were then cultured for another 48 h without PMA, plated at the indicated density, washed with serum free medium or PBS as indicated to remove nonadherent cells. The adherent cells (macrophages) were treated with recombinant human TFPI-2 (rTFPI-2, R&D System) in different concentrations.

2.2. Cell proliferation assay

After U937-derived macrophages were seeded in a 96-well plate, cells were treated with rTFPI-2 (0, 1.25, 2.5, 5.0, and 10.0 μg/ml) for 48 h. Cell proliferation was assessed by measuring 5-bromo-2-deoxyuridine (BrdU) (Sigma) incorporation during DNA synthesis in proliferating cells. Cells are fixed with 4% paraformaldehyde for 30 min at 4 °C, and then cells were processed through a series of staining and washing: washed 3 min in PBS with 1% Triton X-100 and 1% dimethyl sulfoxide (DMSO) (TD buffer), placed in acetone (−20 °C) for 3 min, washed in TD buffer for 3 min, placed in 0.3 N HCl for 3 min, washed in TD buffer for 3 min, incubated in whole goat serum 1:10 for 30 min at 37 °C, and stained overnight in monoclonal anti-BrdU antibody (1:30) and washing buffer at room temperature with agitation (100 turns per minute). The second day, cells were washed with TD buffer three times for 30 min and placed in FITC-conjugated goat anti-mouse secondary antibody (1:20) overnight at room temperature with agitation (100 turns per minute). The BrdU labeling indices were assessed by counting the nuclei through a microscope. The labeling index was expressed as the number of positively labeled nuclei/total number of nuclei × 100%. Triplicate wells were assessed for each treatment, experiments were done three times. Results were compared with control cells.

2.3. DNA fragmentation determination

For detection of apoptosis, a sandwich, one step, colorimetric enzyme-linked immunosorbent assay (ELISA), the Cell Death Detection ELISA Plus kit (Roche Diagnostics, Germany) was used. The assay allows for the specific determination of histone-complexed DNA fragments (mono and oligonucleosomes) from the cell cytoplasm, after the induction of apoptosis. Briefly, after induction of apoptosis and 48 h incubation, the cells were pelleted by centrifugation (200 *g*, 10 min) and the supernatants (containing DNA from necrotic cells that leaked through the membrane during incubation) were discarded. Cells were resuspended and incubated for 30 min in lysis buffer. After lysis, intact nuclei were pelleted by centrifugation. Aliquots of the supernatants were transferred to a streptavidin-coated well of a microtiter plate with two monoclonal antibodies, antihistone (biotin-labeled) and anti-DNA (peroxidase-conjugated), so nucleosomes in the supernatant created antibody–nucleosome complexes, which continuously were bound to the microtiter plate by streptavidin. All samples were then incubated with peroxidase substrate and absorbance was measured at 405 nm. Triplicate wells were assessed for each treatment, experiments were done three times. The mean of optical densities of three different controls was considered to be 100% and all other values were expressed as % of the controls.

2.4. Caspase activity assay

Caspase-3 and caspase-9 activities were detected using Caspase-3 Assay Kit and Caspase-9 Assay Kit (Millipore, USA) respectively, according to the manufacturer's directions. The assays are based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the labeled substrates DEVD-pNA (caspase-3) and LEHD-pNA (caspase-9), respectively. Briefly, U937-drived macrophages were incubated in 6-well plates for 48 h with the final rTFPI-2 concentrations of 1.25, 2.5, 5 and 10 μg/ml. A control without drug treatment was incubated in the mean time. 5×10^6 cells were suspended in 50 μl of chilled cell lysis buffer and incubated cells on ice for 10 min. After centrifugation (5 min, 10,000 × *g*), supernatants (cytosolic extracts) were transferred to a fresh tube and put on ice. The protein concentration for each sample set was assayed by BCA method (BCA™ protein assay kit, Pierce). Samples were incubated with the substrates for 2–3 h at 37 °C and measured at 405 nm, as indicated. The absorbance of pNA from every sample was compared with the control and values were expressed as micromoles of pNA per microgram of cytosolic protein (μM/μg). Triplicate wells were assessed for each treatment and experiments were done three times.

2.5. Hoechst 33342 assay

U937-derived macrophages were seeded in a 24-well plate and treated with rTFPI-2 (0, 1.25, 2.5, 5.0, and 10.0 μg/ml) for 48 h. After treatment, cells were fixed, washed twice with PBS and stained with Hoechst 33342 staining solution for 5 min at room temperature and observed under fluorescence microscope using a 4′,6-diamidino-2-phenylindole (DAPI) filter. Fragmented or condensed nuclei were scored as apoptotic.

2.6. Double staining of annexin V and PI

U937-derived macrophages were seeded in a 6-well plate and treated with rTFPI-2 (0, 1.25, 2.5, 5.0, and 10.0 μg/ml) for 48 h. Then cells were stained with annexin V and PI and then evaluated for apoptosis by flow cytometry according to the manufacturer's protocol (BD Biosciences). Briefly, at the end of the co-incubation period, cells were collected by trypsinization, washed twice with cold PBS, and centrifuged at 2000 rpm for 5 min. Then cells were resuspended in 400 μl binding buffer at a concentration of 1×10^6 cells/ml, 100 μl of the solution was transferred to a 5 ml culture tube, and 5 μl of annexin V and 5 μl of PI were added. Cells were vortexed gently and incubated for 15 min at room temperature away from light. Finally, 400 μl of binding buffer was added to each tube, and samples were analyzed by FACScan flow cytometer. For each sample, 10,000 ungated events were acquired.

Cells in the lower right quadrant correspond to early apoptotic cells (annexin V-positive and PI-negative), whereas those in the upper right quadrant correspond to late apoptotic or necrotic cells (annexin V-positive and PI-positive).

2.7. Western blot analysis

U937-derived macrophages were seeded in 6 cm dishes and treated with rTFPI-2 (0, 1.25, 2.5, 5.0, and 10.0 μg/ml) for 48 h. After co-incubation, the culture supernatants were collected, centrifugated (5 min, 12,000 rpm) and transferred to a fresh tube on ice for FasL analysis. Then cells were collected by trypsinization, washed three times with PBS and extracted in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 1% NP40, 0.1% SDS, 1 mM PMSF, 1 mg/ml each of aprotinin, leupeptin, and pepstatin) for 10 min. The lysates were centrifuged at 12,000 rpm for 5 min at 4 °C, and the supernatant was collected for Fas or caspase-8 analysis. Protein concentration was determined by BCA method. Approximately 60 μg protein from each culture supernatant or cell extract was separated by electrophoresis on 10% SDS-polyacrylamide gels and then transferred to polyvinyl difluoride (PVDF) membrane (Ame rsham Biosciences Europe). PVDF membranes were blocked in blocking solution (50 mM Tris–HCl, 150 mM NaCl, 5% (w/v) non-fat dry milk and 0.1% Tween 20) overnight at 4 °C. The primary antibodies (diluted 1:400) and a second antibody consisting of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (diluted 1:2,000) were used for the detection of active caspase-8 (BioVision), Fas and FasL (Santa Cruz Biotechnology).

2.8. Statistics

All the experiments were formed as triplicate measurements in three parallel wells and the results were shown as the mean ± S.D. Statistical differences were evaluated using the Student's t-test. Differences between means were considered significant when $P<0.05$ or $P<0.01$.

3. Results

3.1. Effect of rTFPI-2 on U937-derived macrophages proliferation

To demonstrate whether rTFPI-2 affects U937-derived macrophages proliferation, we applied BrDU incorporation assay. As is shown in Fig. 1A and B, the rate of BrdU incorporation into U937-derived macrophages was slightly decreased in rTFPI-2-treated macrophages. However, there was no significant difference between rTFPI-2-treated and control cells ($P>0.05$; Fig. 1B). This implied that rTFPI-2 had minor effects on macrophages proliferation.

3.2. Detection of DNA fragmentation of apoptotic cells

A significant increase of apoptotic DNA fragments was noted after 48 h treatment of U937-drived macrophages with 1.25 μg/ml rTFPI-2 (257.75 ± 22.01%), 2.5 μg/ml rTFPI-2 (470.78 ± 27.39%), 5.0 μg/ml rTFPI-2 (609.76 ± 38.20%), 10.0 μg/ml rTFPI-2 (626.22 ± 45.11%), compared to control (100 ± 7.04%) (Fig. 2). The results indicated that rTFPI-2 promoted U937-derived macrophages apoptosis with the increase of rTFPI-2 concentration.

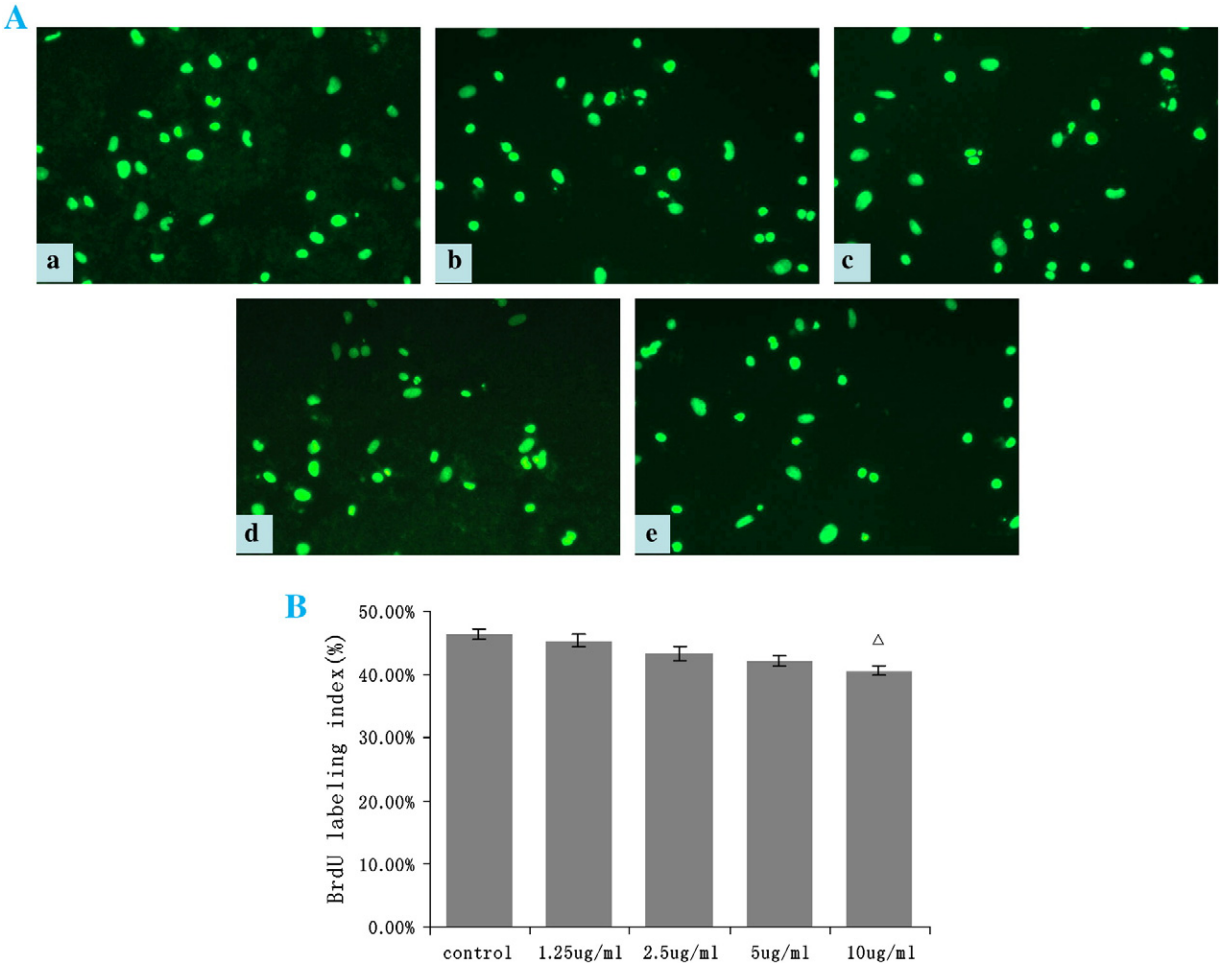

Fig. 1. Effect of rTFPI-2 on U937-derived macrophages proliferation. Cells were seeded at a 96-well plate and treated with rTFPI-2 (0, 1.25, 2.5, 5.0, and 10.0 μg/ml) for 48 h. Subsequently, cells were incorporated by BrdU and then treated with FITC-conjugated goat anti-mouse secondary antibody to visualise the anti-BrdU labeled cells (A). Each bar or point indicates the mean of the BrdU labeling index. Results are means ± S.D. of three independent experiments. △$P>0.05$, compared with the control (B).

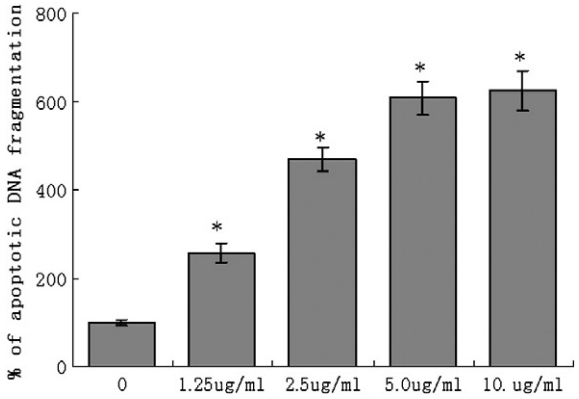

Fig. 2. Colorimetric enzyme-linked immunosorbent assay for detection of DNA fragmentation of apoptotic cells. Assessment of apoptotic DNA fragmentation revealed that U937-derived macrophages treated with rTFPI-2 resulted in a concentration-dependent induction of apoptosis. Results are means ± S.D. of three independent experiments. *P<0.05, compared with the control.

3.3. Measurement of caspase activity

The colorimetric evaluation of caspase-3,9 activity was made after 48 h incubation of U937-drived macrophages with rTFPI-2. At the concentration of 1.25 μg/ml, rTFPI-2 induced significant increase of activities of caspase-3 (3.89 ± 0.24 μM/μg), caspase-9 (2.59 ± 0.26 μM/μg), compared to controls (2.22 ± 0.15, 1.56 ± 0.13 μM/μg, respectively). With the concentration of rTFPI-2 raised (2.5, 5.0 and 10.0 μg/ml, respectively), a gradual increase of caspase-3 (6.07 ± 0.42, 5.72 ± 0.40, 7.29 ± 0.52 μM/μg, respectively) and caspase-9 (3.70 ± 0.31, 3.79 ± 0.30, 4.61 ± 0.34 μM/μg, respectively) was observed. (Fig. 3).

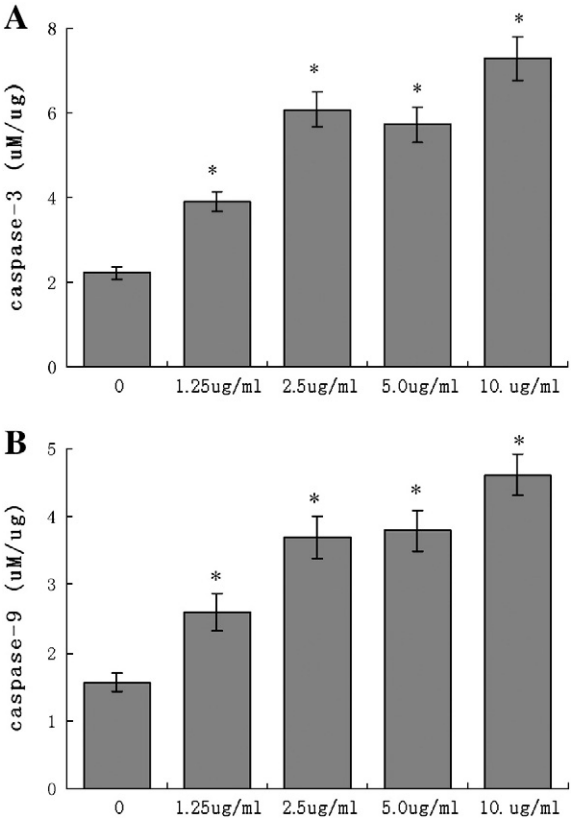

Fig. 3. Measurement of caspase-3 (A) and caspase-9 (B) activities. After 48 h incubation of U937-drived macrophages without or with 1.25, 2.5, 5.0, and 10.0 μg/ml of rTFPI-2, the colorimetric evaluation of caspase-3,9 activities were made. Results are means ± S.D. of three independent experiments. *P<0.05, compared with the control.

3.4. rTFPI-2 induces macrophages apoptosis in a dose dependent manner

In order to verify whether the induction of apoptosis occurred during rTFPI-2 treatment, the presence of apoptotic cells was determined by means of Hoechst 33342 staining, and the application of double staining with annexin V and propodium iodide. The cell morphology observation in Fig. 4A suggested that exposure of rTFPI-2 to macrophages produces an apoptotic effect. In addition, flow cytometry assays (Fig. 4B) revealed that treatment of macrophages with various concentrations of rTFPI-2 for 48 h caused remarkable apoptosis versus control with a quantitative characterization of the effect (Fig. 4C). All these results were consistent with those we found in Hoechst 33342 assay. Taken together, these results indicated that rTFPI-2 induced U937-derived macrophages apoptosis in a dose dependent manner.

3.5. rTFPI-2 upregulates Fas/FasL activation and promotes caspase-8 activation

Death receptors of the TNF family such as Fas are the best-understood death pathways that recruit Fas-associated death domain (FADD) and procaspase-8 to the receptor in macrophages. Recruitment of procaspase-8 through FADD leads to its auto-cleavage and activation, and in turn activates effector caspases such as caspase-3 in causing cell death (Liu et al., 2009). Western blot analyses displayed an increase of Fas protein expression in cell extracts after rTFPI-2 treatment for 48 h (described in Fig. 5A), so did the FasL protein expression in culture supernatants (described in Fig. 5B). Moreover, rTFPI-2 induced the expression of Fas and FasL in a concentration-dependent manner.

Caspases, a family of cysteine protease, are the central components of apoptotic response. Caspases are a conserved family of enzymes that irreversibly commit a cell to die. They are synthesized as latent zymogens organized in cascade system that upon activation stimulate apoptosis. If the program of apoptosis is stimulated, caspases will be activated (Bhattacharyya et al., 2003). Western blot analysis for cleaved, active caspases was undertaken to determine caspase activation. As shown in Fig. 5C, Western blot analyses also displayed an increase in a dose-dependent manner in caspase-8 protein expression after rTFPI-2 treatment.

Together, these findings suggest that TFPI-2 induced cell apoptosis is related to autocrine Fas-mediated apoptosis pathway activation.

4. Discussion

TFPI-2 is a protease inhibitor that could play a regulatory role in several processes that are relevant to the physiology of healthy vessels and to the pathogenesis of atherosclerosis. In vitro, TFPI-2 can inhibit extracellular proteolytic activity and TF-mediated coagulation and may also influence cell migration and proliferation. Dysfunction of these processes has been reported to contribute to atherogenesis.

Although macrophages are relatively long-lived cells, there is a finite incidence of macrophage apoptosis throughout atherogenesis. The functional consequence of early lesional foam cell formation on atherogenesis has been debated (Steinberg, 1983), but genetic studies in mice in which monocytes are depleted or monocyte entry into lesions is blocked suggest that these macrophages contribute to the progression of atherosclerosis (Smith et al., 1995). Moreover, once early lesional macrophage apoptosis was blocked by genetic engineering in mice, the lesions became more cellular and atherogenesis was accelerated (Liu et al., 2005). When early lesional macrophage apoptosis is enhanced, a decrease in lesion progression has been

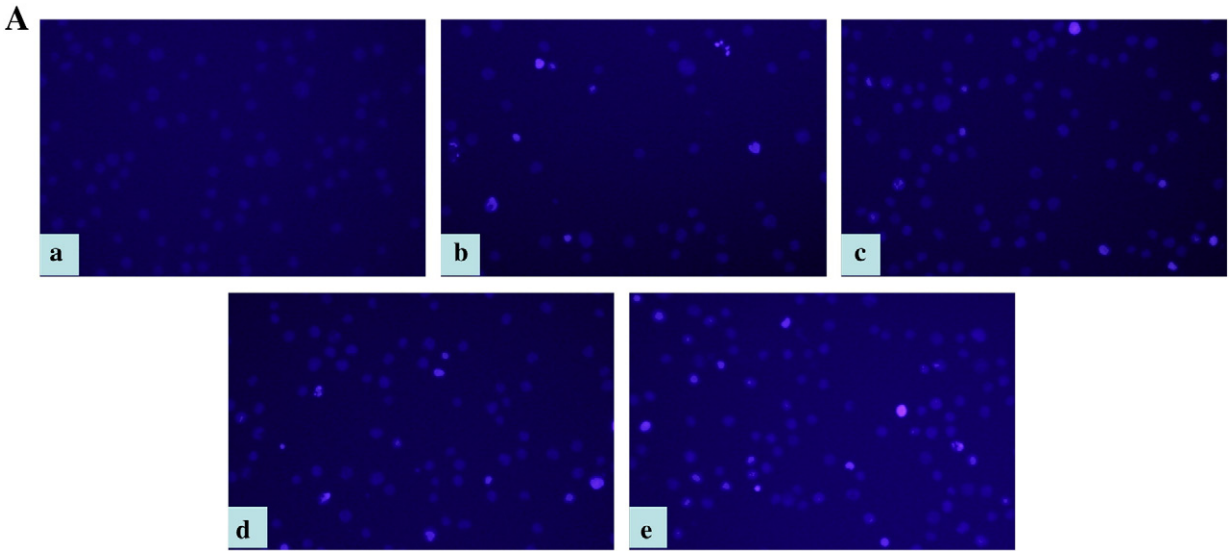

Fig. 4. A. Morphological effect of TFPI-2 on the apoptosis in macrophages. Monolayers of macrophages were incubated without (a) or with 1.25 (b), 2.5 (c), 5.0 (d), 10.0 μg/ml (e) of rTFPI-2 for 48 h. At the end of the incubation, the cells were washed, fixed, and stained with Hoechst 33342. All fields were representative of multiple fields observed in three independent experiments (original magnification ×400). B. Double staining of annexin V and PI. Cells were treated without (a) or with 1.25 (b), 2.5 (c), 5.0 (d), 10.0 μg/ml (e) of rTFPI-2 for 48 h. The presence of apoptotic cells was identified by flow cytometric analysis of cells labeled with annexin V and PI. Cells in the lower right quadrant correspond to early apoptotic cells, whereas those in the upper right quadrant correspond to late apoptotic or necrotic cells. Numbers in each quadrant are percentage of cells they contain. The results shown are representative of three separate experiments. C. Quantitation of macrophages apoptosis induced by rTFPI-2. Cells were treated without (control) or with 1.25, 2.5, 5.0, 10.0 μg/ml of rTFPI-2 for 48 h. The cells were collected, fixed, and sent to analyze for apoptotic ratio by flow cytometry. Each bar or point indicates the mean of cell apoptosis ± S.D. *P<0.05 versus control.

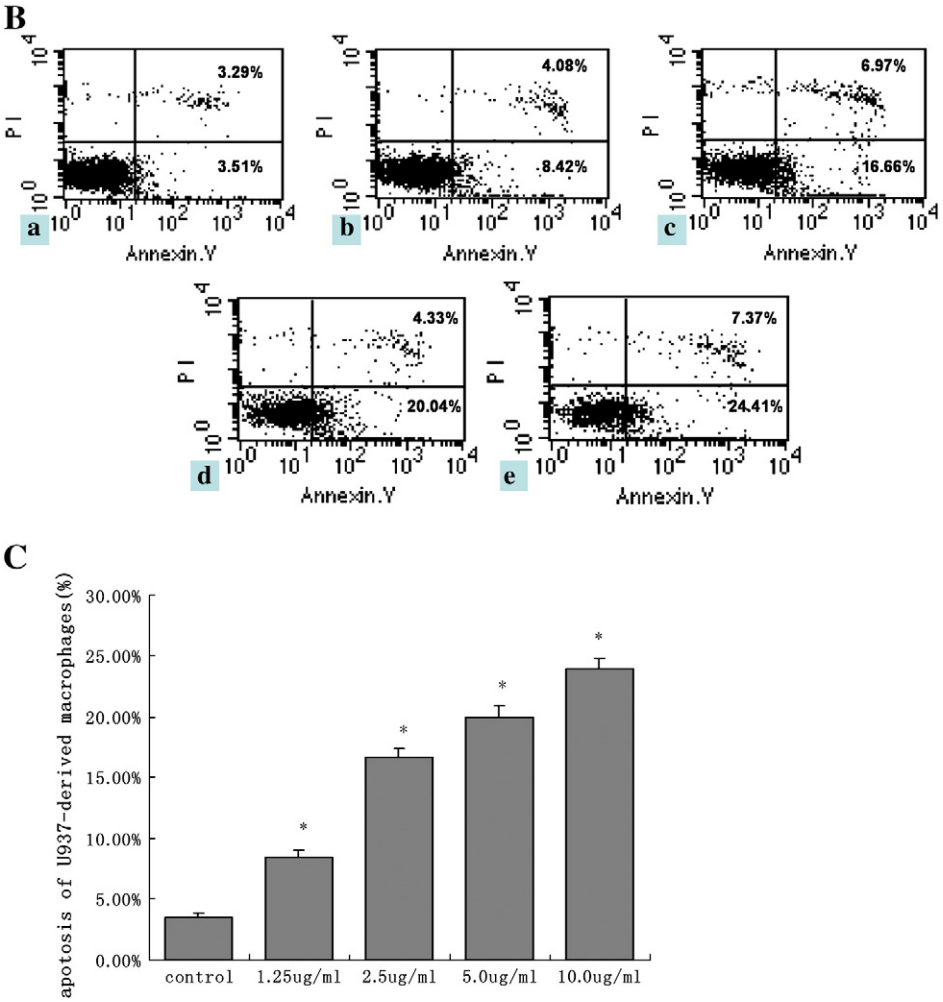

Fig. 4 (*continued*).

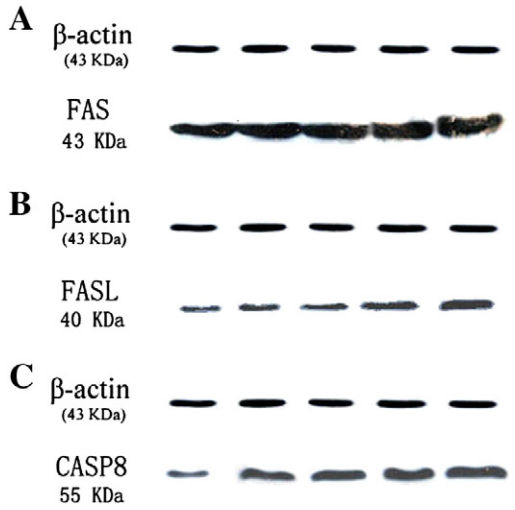

Fig. 5. Activation of caspase 8 in macrophages after rTFPI-2 incubation. Monolayers of macrophages were incubated without or with rTFPI-2 in different doses (1.25, 2.5, 5, and 10 μg/ml) for 48 h. At the end of incubation, the cells were collected and lysed. Equal amounts of protein (60 μg) were subjected to SDS-PAGE followed by Western blot analysis with anti-Fas (A) or FasL (B), caspase 8 (C) antibody. The blots were stripped and reprobed with a β-actin-specific antibody as a measure of equal loading.

observed (Arai et al., 2005; Tabas, 2005). These data further support the notion that macrophages play a pro-atherogenic role and suggest that the turnover of macrophages by apoptosis limits lesion cellularity in early atherogenesis. Our results showed that rTFPI-2 could promote macrophages apoptosis markedly, which indicates TFPI-2 may limit lesion cellularity in early atherogenesis.

In normal physiology, during which apoptosis occurs at a very high rate with estimates as high as hundreds of billions of cells per day, apoptotic cells are rapidly cleared by phagocytes. Importantly, the clearance of apoptotic cells elicits an active anti-inflammatory response in efferocytes (Fadok et al., 1998). Thus, effective phagocytosis, together with evidence indicating that living macrophages promote plaque progression (Smith et al., 1995; Boring et al., 1998; Gu et al., 1998), likely explains why increasing early lesional macrophage apoptosis has antiatherogenic consequences, and vice versa (Kockx and Herman, 2000).

Activation of caspases is achieved via two principal signaling pathways, namely the extrinsic and intrinsic death pathways (Schulze-Osthoff et al., 1998; Li and Yuan, 1999). The extrinsic death pathway involves the ligation of death receptors (CD95/Fas; tumor necrosis factor (TNF) receptor) that leads to the recruitment of adaptor molecules such as Fas-associated death domain (FADD), and pro-caspase 8 or procaspase 10 into a death-inducing signaling complex (DISC) (Kischkel et al., 1995; Wang et al., 2005). FasL plays important roles by rapidly inducing apoptosis under numerous physiologic and pathologic conditions (Wajant, 2006). Our data suggest that Fas/FasL pathway was possibly involved in apoptosis of U937-derived macrophages and enhanced by rTFPI-2. In the future study we will further identify the effect of this pathway in rTFPI-2 induced apoptosis in macrophages and identify whether TFPI-2 has antiatherogenic effects in vivo.

However, there are some limitations in our study. Firstly, we just assessed the FasL expression in the culture supernatants not in the condition media, while an increase of the FasL expression in the culture supernatants cannot be the direct evidence that rTFPI-2 promotes apoptosis through FasL. Secondly, our results of the present study were from U937-derived macrophages, and further study is still needed to corroborate in human (fresh) monocyte-derived macrophages. Lastly, further in vivo study is necessary to explore TFPI-2 on antiatherogenic effect and elucidate the mechanisms involved.

5. Conclusions

In summary, the present data provide evidence for the first time that extrinsic rTFPI-2 induces apoptosis in cultured U937-derived macrophages. The apoptotic effect is possibly through Fas/FasL pathway to activate caspase-8. Therefore, rTFPI-2 may provide a new method to treat atherogenesis. Further studies are needed to elucidate the mechanism of rTFPI-2 on induction of apoptosis in detail. Furthermore, studies are needed to identify on whether TFPI-2 has antiatherogenic effects in vivo.

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