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Alternatively Spliced Tissue Factor and Full-length Tissue Factor Protect Cardiomyocytes against TNF-α-induced Apoptosis

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Abstract

Tissue Factor (TF) is expressed in various cell types of the heart, such as cardiomyocytes. In addition to its role in the initiation of blood coagulation, the TF:FVIIa complex protects cells from apoptosis. There are two isoforms of Tissue Factor (TF): “full length” (fl)TF – an integral membrane protein; and alternatively spliced (as)TF – a protein that lacks a transmembrane domain and can thus be secreted in a soluble form. Whether asTF or flTF affect apoptosis of cardiomyocytes is unknown.

In this study, we examined whether asTF or flTF protect murine cardiomyocytes from TNF-α-induced apoptosis. We used murine cardiomyocytic HL-1 cells and primary murine embryonic cardiomyocytes that overexpressed either murine asTF or murine flTF, and stimulated them with TNF-α to initiate cell death. Apoptosis was assessed by Annexin-V assay, propidium iodide assay, as well as activation of caspase-3 and -9. In addition, signaling via integrins, Akt, NFκB and Erk1/2, and gene-expression of Bcl-2 family members were analyzed.

We here report that overexpression of asTF reduced phosphatidylserine exposure upon TNF-α-stimulation. asTF overexpression led to an increased expression and phosphorylation of Akt, as well as up-regulation of the anti-apoptotic protein Bcl-x_L. The anti-apoptotic effects of asTF overexpression were mediated via $\alpha_V\beta_3$/Akt/NFκB signaling and were dependent on Bcl-x_L expression in HL-1 cells. The anti-apoptotic activity of asTF was also observed using primary cardiomyocytes. Analogous yet less pronounced anti-apoptotic sequelae were observed due to

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Disclosures

None declared.

overexpression of flTF. Importantly, cardiomyocytes deficient in TF exhibited increased apoptosis compared to wild type cells.

We propose that asTF and flTF protect cardiomyocytes against TNF-α-induced apoptosis via activation of specific signaling pathways, and up-regulation of anti-apoptotic members of the Bcl-2 protein family.

Keywords

Apoptosis; Cardiomyocytes; Tissue Factor

1. Introduction

Tissue factor (TF) is the primary initiator of coagulation and is expressed in the heart in various cell types, such as fibroblasts and cardiomyocytes [1–5]. Myocardial TF protects the heart and other organs against hemorrhage [6]. TF is also located in intercalated discs in cardiomyocytes. Moreover, it has been suggested that TF is involved in the maintenance of the structural integrity of the myocardial muscle [7]. Pawlinski *et al*. showed that mice with a selective deletion of the TF gene in cardiac myocytes exhibit increased hemosiderosis and fibrosis after treatment with isoproterenol, indicating that TF plays a protective role under pathological conditions [6].

In 2003, Bogdanov *et al*. described an alternatively spliced TF (asTF) isoform of TF [8]. Low levels of both asTF and flTF isoforms are present in blood [8–10]. The soluble asTF isoform lacks the 5^{th} exon, which results in a frame shift and the loss of the transmembrane and cytosolic domains [8]. asTF has minimal pro-coagulant activity [8,11–15]. During heart development, murine asTF and flTF exhibit similar expression patterns, but asTF is maximally expressed at later stages [9]. flTF protects the heart against intra-cardiac bleeding, although it was speculated that flTF has other roles in the heart tissue, including maintenance of vessel stability [7]. Less is known about the biological function of asTF in the heart. Our studies showed that both TF isoforms, flTF and asTF, were down-regulated in the myocardium of patients with dilated cardiomyopathy [16].

asTF has been shown to have a pro-angiogenic effect in solid tumors [17]. In a recent study, recombinant human asTF was demonstrated to induce angiogenesis non-proteolytically via a mechanism that involves engagement of integrins $\alpha_V\beta_3$ and $\alpha_6\beta_1$ on endothelial cells [13]. flTF also contributes to angiogenesis yet, unlike asTF, this effect is achieved via proteolytic activation of protease-activated receptors (PARs) [18]. We note that flTF has been shown to have both pro-apoptotic and anti-apototic activity, depending on the cell type (19,20,21). We hypothesize that asTF may be an anti-apoptotic factor in the heart tissue.

Adult cardiomyocytes do not proliferate. Therefore, the loss of cardiomyocytes leads to cardiac dysfunction. Protection of cardiomyocytes from apoptosis may ameliorate the outcome of several life-threatening cardiac diseases. Myocardial hypoxia and inflammation as well as hypertrophy are associated with an increased incidence of apoptosis [22–24]. Whether asTF and flTF modulate the survival of cardiomyocytes upon inflammatory

stimulation is not known. Therefore, we sought to determine whether asTF or flTF has an influence on the viability of cardiomyocytes.

2. Methods

2.1. Overexpression of asTF and flTF in HL-1 cells

HL-1 – murine cardiomyocytic cells [25] – were kindly provided by Prof. Claycomb (Louisiana State University Health Sciences Center, New Orleans, LA, USA). HL-1 cells were transfected with the murine asTF-plasmid or murine flTF-plasmid (containing the complete coding sequence including the signaling peptide, beginning from the start codon until the stop codon) using Lipofectamine^{2000} (Invitrogen) according to the manufacturer's instructions. As a control, HL-1 cells were transfected with an empty plasmid. For stable transfection, the transfected cells were cultured in Claycomb medium [25] supplemented with G418 (800 μg/mL, PAA) to select transfected cells for several passages. The mRNA expression of the TF isoforms were measured by semi-quantitative RT-PCR (see Table 1) and by Western blot, using the rabbit polyclonal antibody that detects only the murine asTF isoform [9] (kindly provided by Prof. V.Y. Bogdanov, University of Cincinnati College of Medicine, The Vontz Center for Molecular Studies, Cincinnati, OH, USA), or the murine flTF-specific antibody [9,15]. To ensure that the effects observed in TF isoform-overexpressing, stably transfected HL-1 cells were not due to the disruption of the cardiomyocytic genome, we also utilized transiently transfected cells. Therefore, after the transfection of cells with TF-isoform-containing plasmids or empty controls, respectively, the transfected cells were cultured for 48 h in Claycomb medium [25]. Transfected cells were treated as described below.

2.2. Stimulation of HL-1 cardiomyocytes with TNF-α or Camptothecin (CPT)

TNF-α as well as Camptothecin (CPT) are known to induce apoptosis in several cells, such as cardiomyocytes [26–28]. TNF-α was demonstrated to induce apoptosis of cardiomyocytes via caspase-3 activation [26]. CPT binds and stabilizes DNA topoisomerase I/DNA complexes, leading to DNA breaks and damage. This process results in the activation of caspase-3 and, subsequently, induction of apoptosis in cardiomyocytes [27,28]. TNF-α- and CPT-induced apoptosis were shown to be associated with an alteration of the ratio of pro-apoptotic and anti-apoptotic members of the Bcl-2 family [26,27]. We used TNF-α as well as CPT to induce experimental apoptosis in cardiomyocytes.

HL-1 sub-lines were grown to confluence and, after serum starvation, cells were treated with different concentrations of TNF-α (PeproTech) or CPT (Calbiochem) for 16 h or 5 h, respectively. Protein expression was measured by Western blot analyses using specific antibodies: anti-TF_{FL294} (1:1000) and anti-β-actin (1:10000) obtained from Santa Cruz, anti-active caspase-3 (anti-cleaved caspase-3; 1:500), anti-pErk1/2 (1:1000), anti-pAkt (Ser_{473}; 1:500), anti-Akt (1:1000), anti-NFκB (p65; 1:1000) and anti-p65 (Ser_{536}; 1:1000) obtained from Cell Signaling Technology, anti-GAPDH (1:10000, Calbiochem) and anti-Bcl-x_L antibody (1:500, BD Pharmingen). Secreted proteins were detected by Western blotting post precipitation of total proteins from the supernatant by trichloroacetic acid (10 %; Carl Roth GmbH + Co. KG). mRNA expression levels were determined by semi-quantitative RT-PCR

(see Table 1). Densities of bands were quantified using Gel-Pro 4 analyzer software (Media Cybernetics).

2.3. Apoptosis Assay of HL-1 cells

Stimulated and non-stimulated cells were gently harvested and Annexin-V (AV)/propidium iodide (PI) assay (MoBiTec) was performed according to the manufacturer's instructions. The percentages of AV-positive and PI-positive cells were measured by flow cytometry (FACScan®, Becton Dickinson).

2.4. Inhibition of the Akt/NFκB pathway (integrin-mediated signaling)

HL-1 cells were grown to confluence and serum-starved overnight. Next day, cells were incubated for 30 min with 2 μM Triciribine (Akt inhibitor [29]) or 10 μM BAY 11-7082 (inhibitor of nuclear factor-kappa B; NFκB), or 100 nM cyclic RGD peptides (cRGD; inhibitor of integrin-$\alpha_v\beta_3$), all purchased from Calbiochem, followed by TNF-α stimulation for 30 min or 16 h (different concentrations) for protein isolation or AV assay.

2.5. Isolation of primary cardiomyocytes and overexpression of asTF

Hearts of C57/BL6 murine embryos (E13.5) were aseptically collected and transferred to 1.5 mL tubes, containing 30 μL ice-cold trypsin/EDTA (0.05%/0.02%; PAA), followed by digestion at 4°C overnight and then for 15 min at 37°C. After that, 1 mL DMEM (without L-glutamine, supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and FBS Gold 10% v/v, PAA) was added to each digested heart. For separation of the different cell types, cardiac cell suspensions were pooled in a T-75 flask and incubated for 1 h at 37°C to allow fibroblasts to attach. Afterwards, the supernatant (containing cardiomyocytes) was transferred to a 12 well-plate (approximately 1.4 hearts/well) pre-coated with fibronectin (5 mg/L in 0.02% (w/v) gelatin, TEBU). 16 h later, cardiomyocytes were adherent and the medium was changed. After 2 days, the medium was supplemented with BrdU (0.1 mmol/L; BD Pharmingen). Transfections with the asTF-, flTF-containing or control plasmids were performed with Lipofectamine^{2000} according to the manufacturer's instructions.

Embryonic cardiomyocytes from mice with a cardiomyocyte-specific TF knockout ($TF^{flox/flox}$/Mlc2vCre) were isolated as described above. Genotyping of each embryo was performed as described [6]. All experiments were approved by the Animal Care and Use Committees of the collaborating institutions in compliance with the National Institute of Health guidelines.

2.6. Stimulation of embryonic cardiomyocytes with TNF-α

Murine embryonic cardiomyocytes were serum-starved for 2 h followed by TNF-α stimulation (50 ng/mL) and collected at different time points. For detection of protein expression, cells were lysed and the lysates were analyzed by Western blot analysis using anti-active caspase-3 and anti-TF_{FL294} antibodies. mRNA expression was determined by quantitative TaqMan®-real time PCR using specific gene expression assays for Bax, Bcl-2, Bcl-x_L, and Akt (Applied Biosystems) normalized against 18S-RNA expression (Applied Biosystems).

2.7. Caspase-9 activity assay

Primary embryonic cardiomyocytes were treated with a truncated ("soluble") form of recombinant murine flTF (100 nM; (r)sTF, R&D) or recombinant murine asTF (100 nM; (r)asTF, a generous gift from Prof. V.Y. Bogdanov, University of Cincinnati College of Medicine, The Vontz Center for Molecular Studies, Cincinnati, OH, USA). The amino acid composition of murine (r)sTF comprises the entire extracellular domain of murine flTF, and lacks the transmembrane and the intracellular c-terminal domains. In addition, HL-1 cells, primary embryonic cardiomyocytes, as well as embryonic TF knockout cardiomyocytes were transfected transiently with asTF, flTF or empty control plasmids. Thereafter, cells were incubated with 50 ng/mL of TNF-α. After 16 h, caspase-9 activity was determined by the caspase-9 colorimetric activity assay kit according to the manufacturer's protocol (Millipore). The assay is based on photometric measurement of the chromophore p-nitroanilide (pNA) at 405 nm using a kinetic ELISA plate reader (Molecular Devices).

2.8. Statistical analyses

All data were expressed as mean ± SEM. Data were analyzed by One-way ANOVA or Student's *t*-test. All probability (p) values ≤ 0.05 were deemed significant.

2.9. Online Supplemental Material

The supplemental text provides details about the generation of asTF-plasmids, Bcl-x_L down-regulation, and immune fluorescence staining.

3. Results

3.1. Overexpression of asTF in HL-1 cells reduces apoptosis

Apoptosis was determined by measuring the percentage of phosphatidlyserine (PS)-positive cells (AV assay), and PI-positive cells. We generated HL-1 cells overexpressing asTF (asTF-cells) (Figure 1a). These cells displayed significantly reduced PS exposure and PI-positivity compared to controls (Figure 1b and c). The apoptosis rate after stimulation with CPT was also reduced in asTF-overexpressing cardiomyocytic HL-1 cells (asTF cells) compared to mock transfected control cells (supplemental Figure S2a and b). Next, we analyzed levels of caspase-3 and 9. Under basal conditions, no active caspase-3 was detected in asTF-cells or control cells (Figure 1d). TNF-α stimulation induced the generation of active caspase-3. Compared to asTF-cells, a higher amount of activated caspase-3 was found in mock-transfected HL-1 cells (Figure 1d). Overexpression of asTF also significantly reduced caspase-9 activity in TNF-α treated HL-1 cells (Figure 1e). flTF-overexpressing HL-1 cells (flTF, Figure 1a) also exhibited reduced caspase-9 activity after TNF-α stimulation compared to stimulated mock transfected cells (Figure 1e).

The impact of pharmacologic inhibitors of the Cdc2-like kinases (Clks) on the TF isoform expression was characterized on mRNA level (supplemental Figure S2d–f). Treatment of HL-1 cells with the pharmacologic Clk inhibitors TG003 or KH-CB19, respectively, reduced the mRNA expression of flTF as well as asTF. The inhibitory effect of the newly developed Clk inhibitor KH-CB19 was higher than the impact of TG003 at the same concentration (10 μM), indicating that Clk-dependent activity of spliceosomal factors plays

an important role in the biosynthesis of flTF and asTF mRNA in murine cardiomyocytes, as we previously showed to be the case for human flTF and asTF mRNA in endothelial cells [15].

3.2. Analysis of signal transduction pathways and expression of Bcl-2 proteins

Levels of phosphorylated and non-phosphorylated forms of Akt and Erk1/2 were examined to evaluate the pathways involved in the asTF-mediated anti-apoptotic effects. Stimulation with TNF-α led to the activation of Erk1/2 in asTF-cells and control cells, no significant differences were observed (Figure 2a). Interestingly, both the total expression and the level of phosphorylated Akt were higher in asTF-cells and remained elevated after stimulation compared to stimulated control cells (Figure 2b). Akt expression was also induced on the mRNA level in asTF-cells compared to control cells (Figure 2c). Due to the observed differences in Akt phosphorylation, we used Triciribine to inhibit Akt phosphorylation. Pre-treatment of asTF-cells as well as control cells with Triciribine led to a reduction of Akt phosphorylation in non-stimulated as well as in TNF-α-treated cells (Figure 2d). Triciribine pre-treatment before TNF-α stimulation abolished the anti-apoptotic effect of asTF (Figure 2e).

Semi-quantitative RT-PCR revealed increased mRNA expression of the pro-survival protein Bcl-x_L in stimulated asTF-overexpressing HL-1 cells, compared to stimulated control cells (Figure 3a). In contrast, mRNA levels of Bcl-2, Bad, or Bax were not affected (Figure 3a). The increased Bcl-x_L mRNA expression in asTF-cells was associated with increased Bcl-x_L protein levels (Figure 3b). siRNA-mediated knockdown of Bcl-x caused a significant reduction of Bcl-x_L expression, and abolished the pro-survival effect of asTF overexpression (Figure 3c). Further, Triciribine treatment reduced Bcl-x_L mRNA expression in asTF-cells to levels in mock control cells (Figure 3d). TNF-α stimulation of asTF-cells, flTF-cells, and mock transfected cells induced mRNA expression of Bcl-x_L (Figure 3e). Inhibition of NFκB with 10 μM BAY 11-7082 reduced the expression of Bcl-x_L in non-stimulated and TNF-α-stimulated cells (Figure 3e). Treatment of HL-1 cells with TNF-α significantly induced NFκB phosphorylation in asTF-, flTF-, and control cells (Figure 3f). Bcl-x_L protein levels were also indcreased in flTF-cells compared to control cells (supplemental Figure S2c). Inhibition of integrin-$\alpha_v\beta_3$ by 100 nM cRGD reduced NFκB phosphorylation in TNF-α-stimulated asTF-cells, but had no significant impact on flTF-cells or control cells (Figure 3f).

3.3. Reduction of apoptosis in asTF-overexpressing primary cardiomyocytes

Next, we overexpressed asTF and flTF in primary embryonic cardiomyocytes (Figure 4a and supplemental Figure S1). We observed increased expression of total Akt, phosphorylated Akt, Bcl-x_L, and Bcl-2 in asTF-overexpressing embryonic cardiomyocytes compared to controls cells (Figure 4b–e). TNF-α treatment resulted in increased caspase-3 activation in mock transfected cardiomyocytes compared to stimulated asTF-overexpressing embryonic cardiomyocytes (Figure 4f). Compared to mock controls, transient transfection of embryonic cardiomyocytes with either asTF- or flTF-expressing plasmids reduced caspase-9 activity 16 h post TNF-α-stimulation (Figure 4g). The observed reduction of caspase-9 activity was greater in asTF-cells compared to flTF-cells (Figure 4g).

Because asTF was found to be secreted into the supernatant of asTF-cells (Figure 1a), we tested the effect of exogenous murine (r)asTF as well as truncated murine flTF – (r)sTF – on caspase-9 activity in embryonic cardiomyocytes. Compared to controls (wt), treatment of cardiomyocytes with either (r)asTF or (r)sTF reduced caspase-9 activity 16 h post TNF-α-induction (Figure 4h). Interestingly, the (r)asTF-treatment after TNF-α stimulation resulted in a significantly greater reduction of caspase-9 activity compared to (r)sTF-treated cardiomyocytes (Figure 4h).

3.4. Increased apoptosis in primary TF-deficient cardiomyocytes

To investigate the effect of reduced levels of TF on apoptosis, embryonic cardiomyocytes were isolated from mice featuring a cardiomyocyte-specific knockout of the TF gene. Compared to cardiomyocytes with normal TF levels, TF knockout cardiomyocytes exhibited reduced expression of total Akt, phosphorylated Akt, Bcl-$x_{L,}$ and Bcl-2 (Figure 5a-d). After stimulation, TF-deficient cardiomyocytes displayed higher levels of activated caspase-3 compared to controls (2.85 ± 0.37 -fold vs. 1.75 ± 0.38-fold, $p<0.05$; Figure 5e). Overexpression of either asTF or flTF significantly reduced the activity of caspase-9 compared to mock-transfected controls (Figure 5f). The caspase-9 activity was significant lower in asTF-cells than in flTF-cells.

4. Discussion

In this study we show that asTF overexpression protects cardiomyocytes against TNF-α- or CPT-induced apoptosis; these anti-apoptotic effects were mediated by Akt, NFκB, and integrin-$\alpha_v\beta_3$ leading to an up-regulation of anti-apoptotic members of the Bcl-2 protein family. Overexpression of asTF led to a significant reduction of PS exposure and caspase-3 activation in HL-1 cells. Both play central roles in the execution phase of apoptosis [30]. These observations are consistent with the results of other groups showing TNF-α as well as CPT to induce caspase-3 activation and subsequently apoptosis in several cell types, such as cardiomyocytes [26–28].

To compare the anti-apoptotic potential of asTF and flTF in cardiomyocytes, we analyzed the effect of asTF and flTF overexpression on the activation of caspase-9, which plays an important role in apoptosis of cardiomyocytes [31]. We found that asTF as well as flTF overexpression reduced caspase-9 activation and TNF-α-induced apoptosis of HL-1 cells. This is consistent with results of other groups showing that flTF protects cells from apoptosis [19,20]. In our study, caspase-9 activity was significantly lower in asTF-cells than in flTF-cells. Similar results were observed using embryonic cardiomyocytes. Vonteo *et al*. found that enterovirus cardiac replication – a process known to induce pro-inflammatory cytokines, such as TNF-α – induces caspase-9 activity and apoptosis in cardiomyocytes from patients with acute myocarditis [31]. In our experiments, we found TNF-α induced the activity of caspase-9. In contrast, Bajaj *et al*. detected no activation of caspase-9 in TNF-α stimulated HL-1 cells [32]. These discrepancies may be due to differences in the experimental setting or culture conditions. Moreover, Bajaj *et al*. used Western blot analyses to measure caspase-9 expression [32]. Here, we used a quantitative method to determine the activity of caspase-9 in TNF-α-stimulated HL-1 cells that might be more sensitive to changes in levels of caspase-9 activation.

To characterize the role of murine asTF or flTF in the absence of endogenous murine TF expression, we performed transfection experiments using TF knockout cardiomyocytes [6]. In these experiments, we observed a greater reduction in caspase-9 activity in cells overexpressing asTF compared with flTF, although we cannot exclude the possibility that there are different expression levels of the two proteins. Finally, the stimulation of cardiomyocytes with (r)asTF or (r)sTF also reduced TNF-α-induced caspase-9 activity. These data suggest that both asTF and flTF exhibit anti-apoptotic activity in murine cardiomyocytes.

The underlying mechanisms of the asTF and flTF pro-survival effects are unknown. flTF protects baby kidney hamster cells and human breast cancer cells, but increases apoptosis via PAR-2 signaling in a keratinocyte cell line [19,20,21]. Erk1/2 [19,33], Akt [20,34], and NFκB [20] were all found to participate in flTF-associated signaling. In this study, HL-1 cells were found to be protected from apoptosis by asTF and flTF. Erk1/2 phosphorylation was induced by TNF-α in control cells as well as asTF-cells, suggesting that Erk1/2 activation is not an asTF-associated mediator. Increased expression as well as a persistent activation of Akt was observed in asTF-cells before and after TNF-α stimulation compared to control cells. In contrast to asTF-overexpressing cells, mock-transfected control cells showed an activation of Akt only post stimulation with TNF-α. Activation of Akt improves cardiomyocyte survival and cardiac remodeling by preventing apoptosis under several pathological conditions, such as ischemia/reperfusion injury and hypoxia [36] [35] [29,37]. In line with these observations, our study showed that pre-treatment of cells with Triciribine inhibited the Akt phosphorylation and abolished the asTF-mediated anti-apoptotic effects. In addition, we found that NFκB inhibition reduced the expression of anti-apoptotic Bcl-x_L in HL-1 cardiomyocytes. These results are consistent with previously reported data showing that NFκB contributes to anti-apoptotic processes and cell survival [20,38,39].

Recently, asTF was found to mediate pro-angiogenic processes and cell migration via integrins in human endothelial cells [13]. Furthermore, integrin signaling was shown to mediate Akt as well as NFκB-dependent cell survival [38,39]. In line with these data, we found cRGD-mediated inhibition of integrin-$\alpha_v\beta_3$ to reduce the TNF-α-induced phosphorylation of NFκB in asTF-cells, but not in control cells or flTF-cells.

Since the Akt pathway impacts mainly the intrinsic apoptosis pathway [40,41], we focused on this pathway, in which the members of the Bcl-2 protein family are implicated. Members of the Bcl-2 family are involved in flTF-dependent anti-apoptotic signaling [19,42]. We demonstrated that Bcl-x_L is an important factor in mediating the asTF- and flTF-associated anti-apoptotic effect in HL-1 cardiomyocytes. Others also demonstrated that TNF-α-induced apoptosis in cardiomyocytes was associated with an alteration in the ratio of pro-apoptotic members, such as Bax, and anti-apoptotic members of the Bcl-2 family, such as Bcl-x_L [26]. Moreover, Borillo *et al*. showed recently that overexpression of the Pim-1 kinase, a downstream effector of Akt-mediated cardio-protection, increased the expression of Bcl-x_L and Bcl-2 in a transgenic mouse model [43]. Together, our data suggest that the TF isoforms mediate anti-apoptotic effects on HL-1 cardiomyocytic cells via Akt and NFκB; in contrast to flTF, asTF increased the expression of Bcl-x_L through NFκB activation via integrin-$\alpha_v\beta_3$ signaling.

Overexpression of asTF in embryonic cardiomyocytes produced anti-apoptotic effects. In isolated embryonic cardiomyocytes as well as HL-1 cells, TNF-α-induced caspase-3 activation was reduced after transfection with the asTF plasmid compared to mock transfected cells. Furthermore, we found elevated expression of the anti-apoptotic proteins Bcl-2 and Bcl-x_L in asTF-overexpressing primary cardiomyocytes. It was previously described that transgenic mice with a heart-specific overexpression of Bcl-2 exhibit a reduced infarct size and fewer apoptotic cells [44]. Further, Bcl-2 blocks p53-mediated apoptosis in cardiomyocytes [45] and increases Bcl-x_L expression, yielding beneficial effects by reducing ischemia/reperfusion injury in rat hearts [46]. In isolated embryonic cardiomyocytes, asTF-overexpression induced the expression of the anti-apoptotic factors Bcl-2 and Bcl-x_L whereas in HL-1 cells, only Bcl-x_L expression was elevated as a result of asTF overexpression. This discrepancy may be due to different developmental stages of the cell types. Nevertheless, asTF-overexpression resulted in an increased expression of anti-apoptotic members of the Bcl-2 family in both settings. Our data allows us to postulate that asTF influences the expression of Bcl-2 proteins.

Reaffirming the association between the asTF level and the survival of the cells, cardiomyocytes possessing a genetic knockout of the TF gene [47] displayed a higher degree of TNF-α-induced apoptosis, and decreased activation of the Akt pathway. Moreover, Bcl-2 and Bcl-x_L expression was reduced in TF-deficient embryonic cardiomyocytes, underscoring the positive association between asTF and the Akt pathway on one hand, and the Bcl-2 protein expression on the other. While the overexpression of asTF as well as flTF in TF-deficient cardiomyocytes reduced caspase-9 activity, the anti-apoptotic activity of asTF-transfected cells was higher compared to cells flTF-transfected cells.

The expression of the TF isoforms can be differentially modulated on the post-transcriptional level by alternative splicing [15, 34]. Regarding the potential physiological relevance of the induced TF isoform expression under pro-inflammatory conditions, it is important to note that asTF and flTF can affect different cellular functions, such as thrombogenicity [15], angiogenesis [13], gene expression [49] or cell survival, as shown in the present work. In this context, we here report that inhibition of Clks reduces the TF isoform expression on the mRNA level in resting as well as TNF-α-induced cardiomyocytes (HL-1 cells). In line with these data, Clks as well as other kinases were demonstrated to regulate the TF isoform expression in TNF-α-stimulated endothelial cells [15, 34]. Therefore, these data indicate that the Clk kinase family is involved in the modulation of the differential TF isoform expression in HL-1 cells.

In conclusion, asTF influences the expression of anti-apoptotic Bcl-2 proteins via the Akt pathway - at least in part - involving NFκB and integrin-$\alpha_v\beta_3$-mediated signaling processes, which results in a reduction of the caspase-cascade and subsequent apoptotic cell death in cardiomyocytes (Figure 6). Therefore, strategies designed to increase myocardial asTF and flTF expression might be an option to treat heart diseases associated with enhanced apoptosis of cardiomyocytes. In particular, the minimally coagulant yet highly anti-apoptotic asTF may comprise a preferred TF isoform whose induction will likely improve heart muscle function, without a significant risk for thrombotic complications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

asTF	alternatively spliced TF
AV	annexin-V
Bad	Bcl-2-Antagonist of Cell Death
Bax	Bcl-2-associated X
Bcl-2	B-cell lymphoma 2
Bcl-x_L	B-cell lymphoma-extra large
CPT	Camptothecin
Clk	Cdc2-like kinases
cRGD	cyclic arginine-glycine-aspartate peptides
DAPI	4′,6-diamidino-2-phenylindole
Erk	extracellular-signal regulated kinases
flTF	full length TF
FVII/a	factor VII/a
FX	factor X
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HL-1	murine immortalized cardiomyocytic cell line
MAP kinase	mitogen-activated protein kinase
PAR-2	protease-activated receptor 2
PI	propidium iodide
PKB/Akt	protein kinase B
TF	Tissue Factor

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Highlights

- asTF and flTF protect cardiomyocytes against TNF-α-induced apoptosis.
- asTF exhibits a greater anti-apoptotic potential than flTF.
- The anti-apoptotic effects of asTF are mediated via Akt, NFκB, Integrin-$\alpha_V\beta_3$ signaling and Bcl-x_L expression.
- TF-deficient cardiomyocytes exhibit increased apoptosis compared with wild type cells.

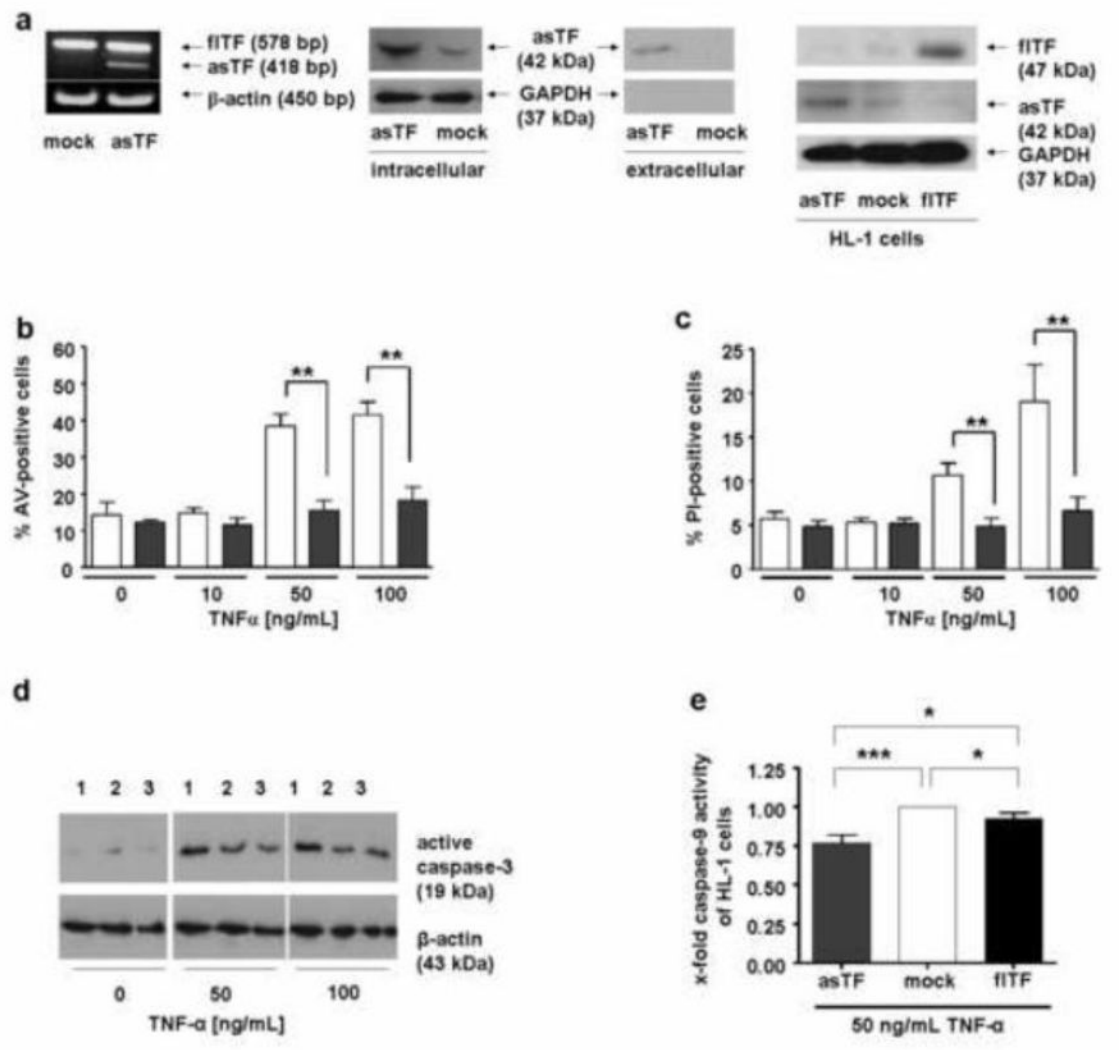

Figure 1. Induction of apoptosis in HL-1 cells

a) Stable overexpression of asTF on mRNA level (left) and protein level (middle left, intracellular; middle right, extracellular = secreted asTF in the supernatant). Right panel shows TF isoform protein expression profile of transiently transfected HL-1 cells. AV-binding (b) and PI-staining (c) assay comparing stable transfected asTF-cells (grey bars) and mock control cells (white bars) stimulated with TNF-α for 16 h (n=6). d) Representative Western blot of active (cleaved) caspase-3 in TNF-α-stimulated stably transfected HL-1 cells post 16 h (1 = controls, 2 and 3: asTF-cells). GAPDH and β-actin were used as loading controls. e) Caspase-9 activity in TNF-α-stimulated HL-1 cells (n=7–9). Compared are cells transiently overexpressing asTF (grey bars), flTF (black bars) and mock-transfected controls (white bars). (***) $p<0.001$, (**) $p<0.01$, (*) $p<0.05$.

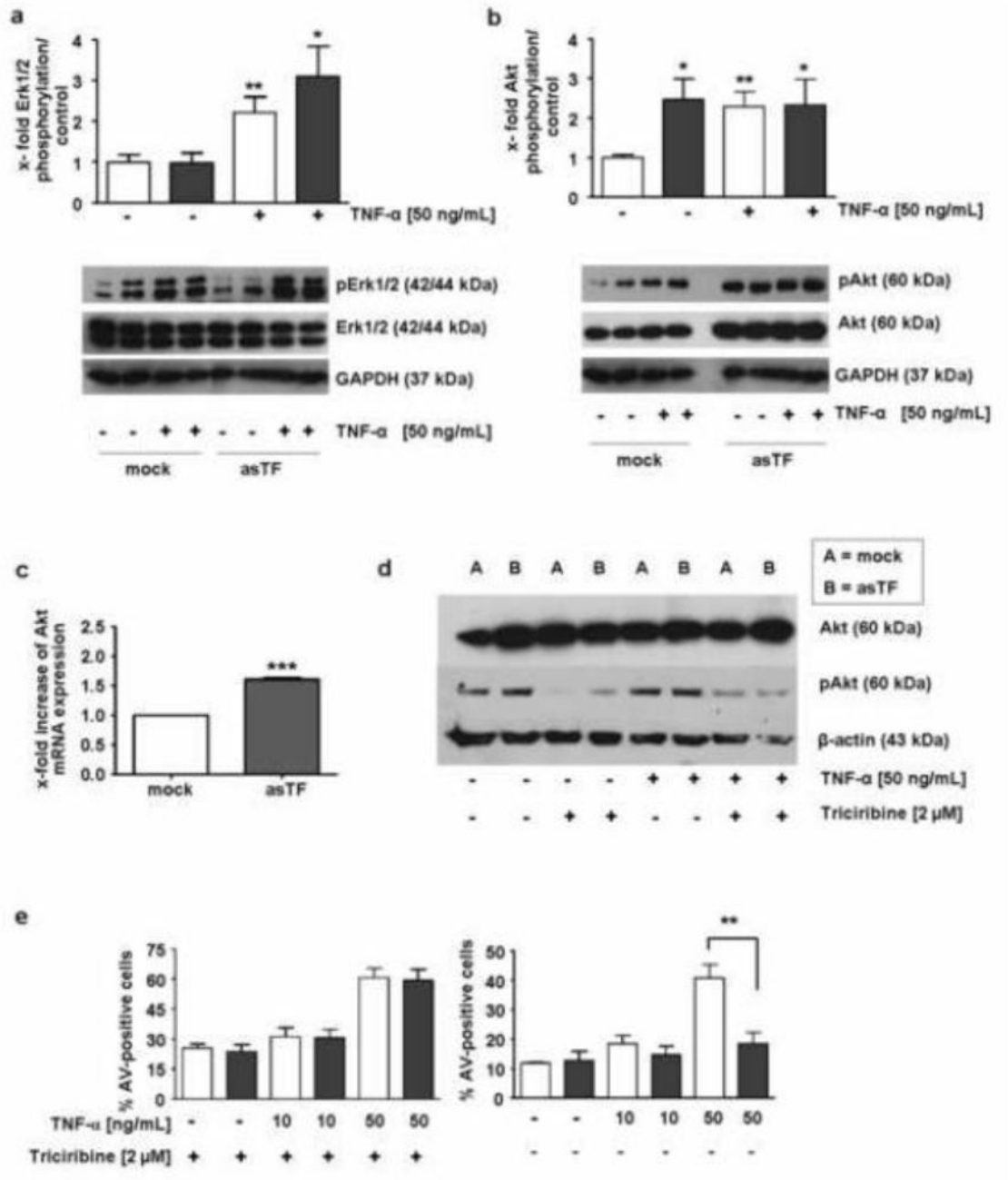

Figure 2. Phosphorylation of Erk1/2 and Akt in HL-1 cells and influence of Akt inhibition on apoptosis

Western blots analyses of a) phosphorylated Erk1/2 and b) phosphorylated Akt 30 min post stimulation with TNF-α in stably transfected cells (controls = white bars; asTF-cells = grey bars; n>6). c) The mRNA expression of total Akt was determined in stably overexpressing asTF-cells or control cells, respectively (n=4). d) Reduction of Akt phosphorylation by Triciribine in non-treated or TNF-α-stimulated stably transfected asTF-cells (asTF) or control cells (mock), respectively. e) AV-apoptosis assay of stably overexpressing cells pre-treated with 2 μM Triciribine and stimulated with TNF-α (n=6). (***) $p<0.001$; (**) $p<0.01$; (*) $p<0.05$.

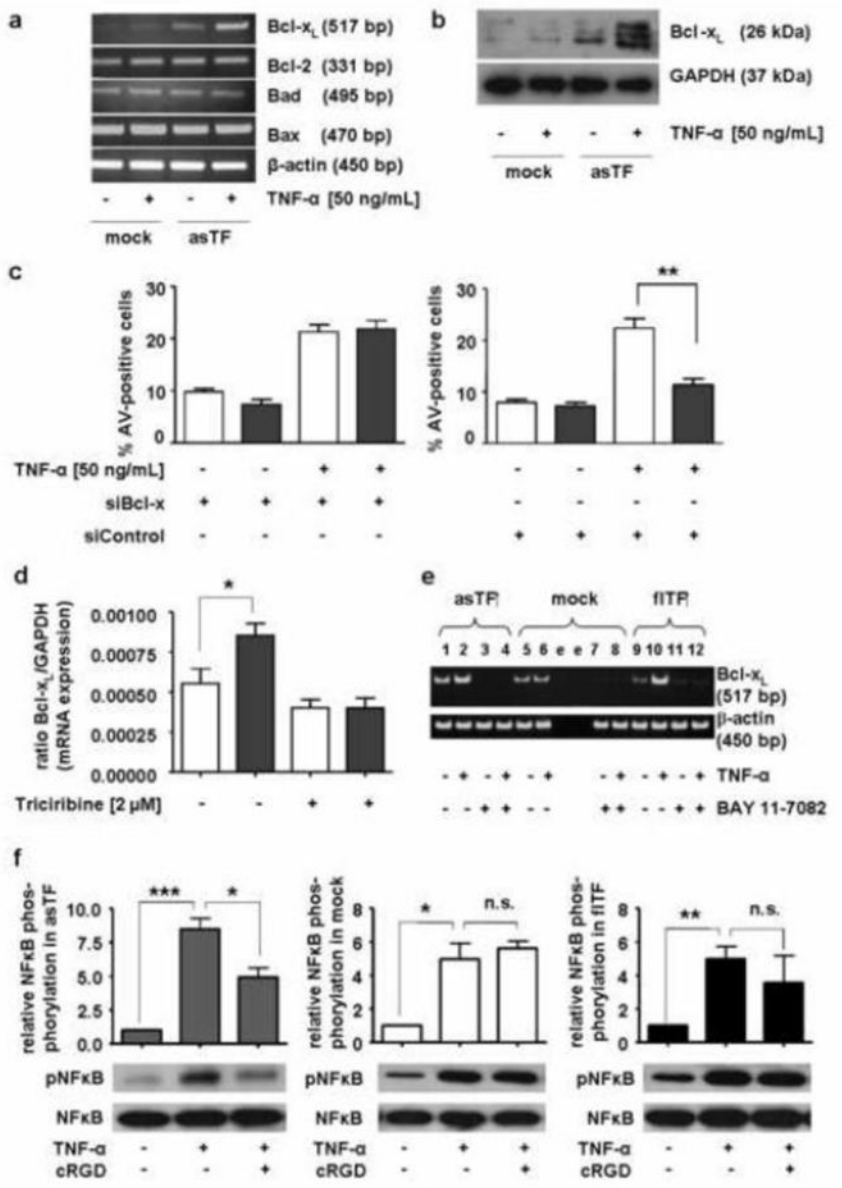

Figure 3. Involvement of Bcl-2 proteins in induced apoptosis in HL-1 cells

Shown is the expression of Bcl-2 family members a) mRNA and b) protein in stably transfected HL-1 cells. a) Compared are control cells (mock) and asTF-cells (asTF) treated with 50 ng/mL of TNF-α for 2 h. b) Protein expression of Bcl-x_L in HL-1 cells (controls and asTF) 6 h post TNF-α stimulation. c) AV-apoptosis assay of stably transfected asTF-cells pre-treated with siRNAs against Bcl-x (siBcl-x) compared to control cells as well as cells pre-treated with control siRNA (siControl; n=8). d) Impact of Triciribine on the mRNA expression of Bcl-x_L in stably transfected HL-1 cells. e) The Bcl-x_L mRNA expression in transiently transfected TNF-α-induced HL-1 cells pre-treated with an NFκB inhibitor (10 μM; BAY 11-7082) post 2 h (1–4 = asTF-cells; 5–8 = mock-transfected cells; 9–12 = flTF-cells; “e” empty lanes; n=3). f) Determination of phosphorylated NFκB (65kDa) in transiently transfected asTF-cells (asTF), flTF-cells (flTF) and control cells (mock) pre-treated with the integrin-$\alpha_v\beta_3$ inhibitor (100 nM; cRGD; n=3). Total NFκB (65 kDa) was used as control. (***) $p< 0.001$; (**) $p<0.01$; (*) $p<0.05$.

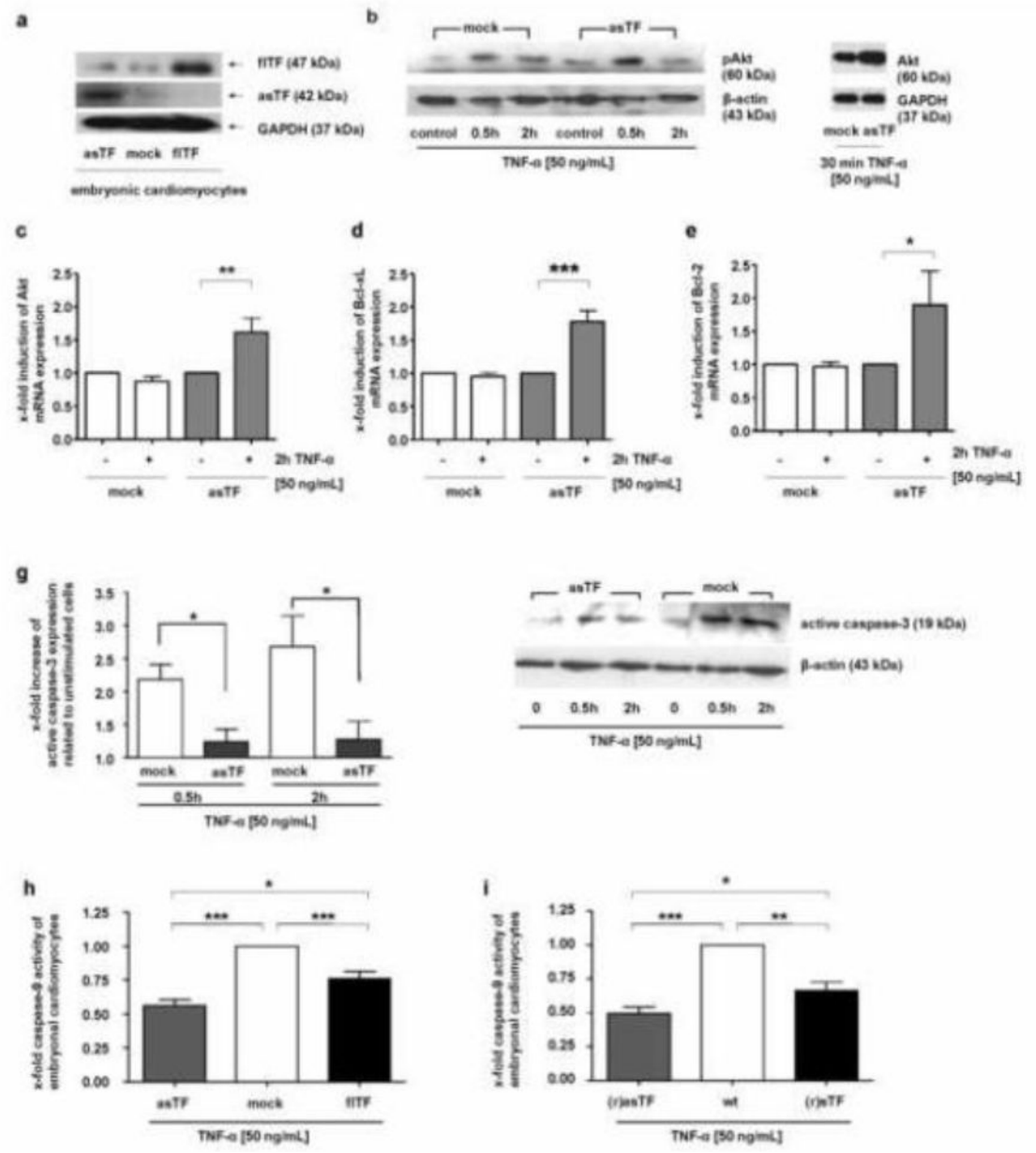

Figure 4. Involvement of Bcl-x_L, Bcl-2, Akt and activation of caspase-3 and caspase-9 in TNF-α-stimulated embryonic cardiomyocytes

a) Protein expression of the TF isoforms in embryonic cardiomyocytes overexpressing asTF (asTF) or flTF (flTF) compared to mock-transfected controls. b) Determination of Akt phosphorylation in asTF-overexpressing embryonic cardiomyocytes compared to controls 0.5 h and 2 h after TNF-α treatment (n=3). Total Akt was also assessed on the protein level in transiently transfected embryonic cardiomyocytes 30 min post TNF-α stimulation. GAPDH or β-actin were used as loading controls. c-e) The mRNA expression of c) total Akt and d) Bcl-x_L and e) Bcl-2 was assessed 2 h post TNF-α stimulation of asTF-cells or control cells, respectively (* $p<0.05$, n=5). f) Shown is the quantification of the relative induction of active caspase-3 expression and a representative Western Blot (n=4). Embryonic cardiomyocytes transfected with asTF (asTF) or the control plasmid (mock) were stimulated with 50 ng/mL TNF-α for 0.5 h and 2 h. g, h) Caspase-9 activity in TNF-α-induced embryonic cardiomyocytes f) transiently transfected with asTF (asTF), flTF (flTF), or empty control vector (mock; n=7–10), and g) cells stimulated with recombinant asTF ((r)asTF) or the soluble extracellular domain of flTF ((r)sTF; n=4) compared to wild type cells (wt). In f and

g, the cells were stimulated with TNF-α (50 ng/mL) for 16 h. In all experiments on isolated primary embryonic cardiomyocytes, transiently transfected cells were used. (***) $p<0.001$; (**) $p<0.01$; (*) $p<0.05$.

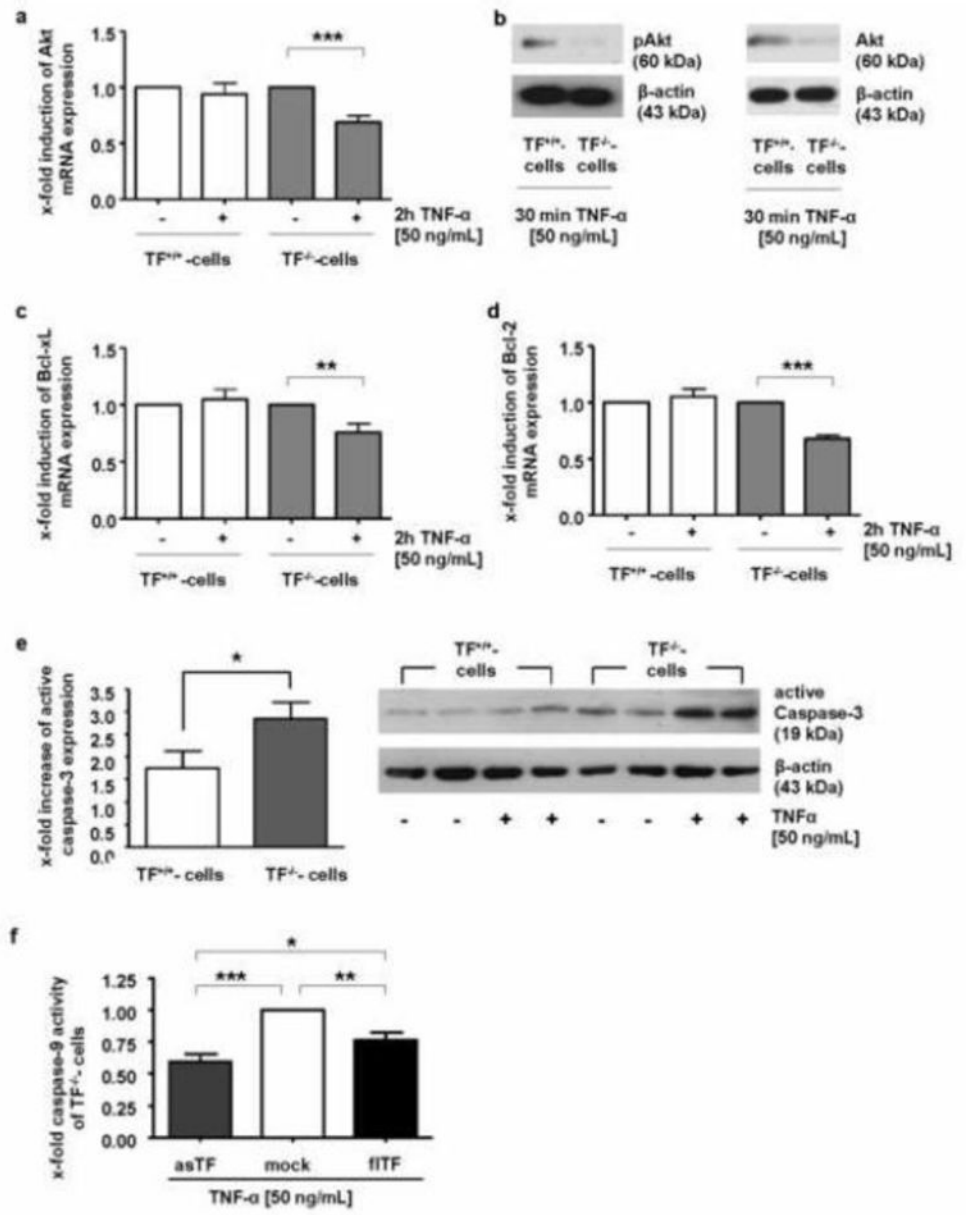

Figure 5. Involvement of Bcl-x_L, Bcl-2, Akt and activation of caspase-3 and caspase-9 in stimulated embryonic cardiomyocytes lacking TF

Shown is (a) Akt mRNA expression, (b) the protein expression and phosphorylation of Akt, (c) Bcl-x_L mRNA expression and (d) Bcl-2 mRNA expression in embryonic TF knockout cardiomyocytes (grey bars, $TF^{-/-}$-cells) compared to control cells (white bar; $TF^{+/+}$-cells) 2 h or 30 min post stimulation with 50 ng/mL TNF-α. e) The increase of active caspase-3 protein of embryonic TF-deficient cardiomyocytes (grey bar, $TF^{-/-}$-cells) compared to control cells (white bar; $TF^{+/+}$-cells) 2 h post stimulation with 50 ng/mL TNF-α (n=4) and a representative Western blots of active caspase-3 and β-actin. f) Caspase-9 activity in embryonic TF knockout cardiomyocytes transiently transfected with asTF (asTF), flTF (flTF) or the empty control plasmid (mock) after 16 h TNF-α stimulation (n=4–5). (***) $p<0.001$; (*) $p<0.05$.

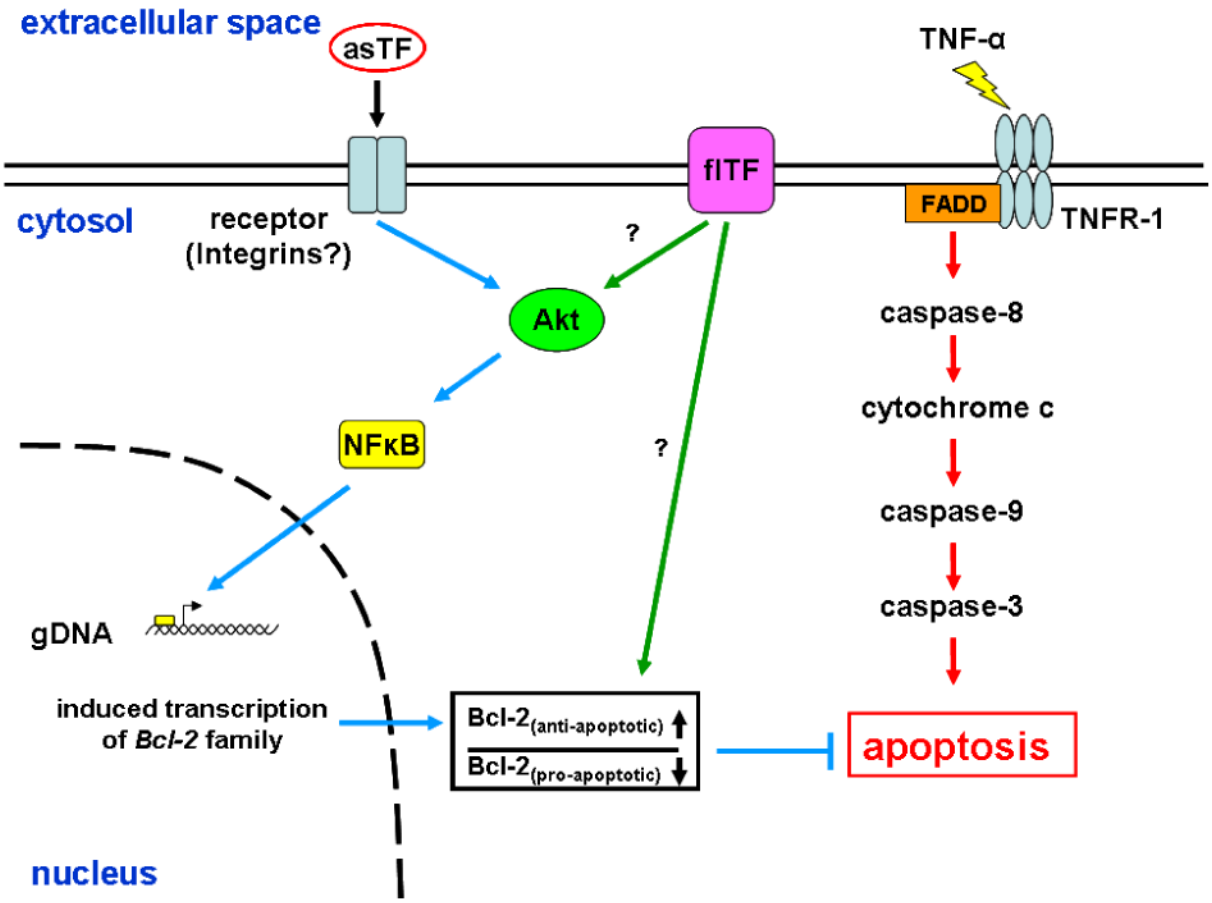

Figure 6. Signaling pathway underlying the anti-apoptotic effect of asTF and flTF in murine cardiomyocytes

Soluble asTF and membrane-bound flTF induces membrane receptor-mediated signaling. Integrins are possible candidates for mediating the effects [48], leading - directly or indirectly - to the activation of Akt, which in turn activates NFκB. Activated NFκB translocates to the nucleus and induces the transcription of its target genes coded within the genomic DNA (gDNA), such as the anti-apoptotic members of the Bcl-2 family (e.g. Bcl-x_L). The induced expression of anti-apoptotic Bcl-2 family members alters the ratio of anti- and pro-apoptotic factors towards an anti-apoptotic state. This prevents the activation of caspase-3 and caspase-9 and, finally, reduces TNF-α induced apoptosis, resulting in increased cell survival of cardiomyocytes.

Table 1

Primer for semi-quantitative PCR

Gene	Forward primer (5′ → 3′)	Reward primer (5′ → 3′)	Tm (°C)
Bax 470bp	ATCGAGCAGGGAGGATGGCT	CTTCCAGATGGTGAGCGAGG	52
Bcl-2 331bp	GTCGCTACCGTCGTGACTTC	AC AGC CAG GAG AAA TCA AAC	52
Bcl-x_L: 517bp Bcl-x_S: 328bp	ACAGCAGCAGTTTGGATGC	ACTGACCGTCCACTCACCTC	52
Bad 495bp	GAGGAAGTCCGATCCCGGAA	CG GCG CTT TGT CGC ATC TGT	52
β-aktin 450bp	AGG GAA ATC GTG CGT GAC AT	TG TCC ACC TTC CAG CAG ATG	52
flTF: 578bp asTF: 418bp	CAAGTGCTTCTCGACCACAGACACC	AGA TGG TGG CCA GGA GCA	52