Overexpression of Alternatively Spliced Tissue Factor Induces the Pro-Angiogenic Properties of Murine Cardiomyocytic HL-1 Cells

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Background: Tissue factor (TF) is expressed in 2 isoforms: membrane-bound “full length” (fl)TF and soluble alternatively spliced (as)TF. flTF is the major thrombogenic form of TF. Although the function of asTF is poorly understood, it was suggested that asTF contributes to tumor-associated growth and angiogenesis. In the heart of a developing embryo, asTF is expressed much later compared to flTF, but in adult heart, asTF exhibits a distribution pattern similar to that of flTF. Thus, it is possible that asTF may play a role in heart development via pro-angiogenic signaling. The purpose of the present study was to examine the effects of murine asTF overexpression in murine cardiomyocyte-like HL-1 cells on their pro-angiogenic potential, the chemotaxis of monocytic cells, and the expression of fibroblast growth factor-2 (FGF2), cysteine-rich 61 (Cyr61), and vascular endothelial growth factor (VEGF).

Methods and Results: Expression of FGF2, Cyr61 and VEGF was assessed on reverse transcription–polymerase chain reaction and western blot. Cell migration, proliferation, and endothelial tube formation assays were carried out. It was found that overexpression of murine asTF in HL-1 cells increases their proliferation and pro-angiogenic properties. The supernatant of murine asTF-overexpressing HL-1 cells induces the chemotaxis of monocytic cells.

Conclusions: Overexpression of murine asTF in murine cardiomyocytic cells increases their proliferation, monocyte migration, and pro-angiogenic properties –possibly– mediated by the induction of the pro-migratory and pro-angiogenic factors FGF2, Cyr61 and VEGF. Thus, we propose that murine asTF may serve as a migration- and angiogenesis-promoting factor. (*Circ J* 2011; **75**: 1235–1242)

Key Words: Alternatively spliced tissue factor; Angiogenesis; Chemokines; Growth; Migration

Ischemic heart disease is a major cause of mortality.¹ Myocardial infarction (MI) is a common representation of ischemic heart disease. Reduced systolic compliance, and arrhythmias due to electrical instability of the myocardium are the 2 main consequences of MI. Therefore, improvement of the blood supply to the infarcted myocardium through the formation of microvessels is a potential therapeutic approach to treat MI.² Angiogenesis as well as cell proliferation are crucial for the regenerative formation of new blood vessels. Both processes are potentiated by various bioactive molecules, such as fibroblast growth factor-2 (FGF2), cysteine-rich 61 (Cyr61, CCN1), and vascular endothelial growth factor (VEGF).^{3–6}

Tissue factor (TF), a 47-kD transmembrane cell-surface glycoprotein, is primarily known as the initiator of the blood coagulation cascade.^{7–11} TF has been detected in the heart, where it is expressed in various subcompartments, for exam-

ple fibroblasts, cardiomyocytes, and cell-derived microparticles.^{7,9,10,12,13} As a result of alternative pre-mRNA splicing, TF is expressed in 2 isoforms: “full length” (fl)TF and alternatively spliced (as)TF.^{14–19} flTF is membrane-bound and comprises the major thrombogenic form of TF.^{7–9} Myocardial flTF protects the heart against spontaneous hemorrhage.²⁰ Our group recently showed that flTF as well as asTF are downregulated in the myocardium of patients with dilated cardiomyopathy.²¹ In contrast to flTF, the biologic function of the soluble asTF isoform is not well understood. Although asTF promotes FXa generation in the presence of phospholipids, the pro-coagulant activity of asTF is much lower than that of flTF.^{8,14,17,22} Recently, it was suggested that human asTF may be linked to increased cell proliferation and angiogenesis.^{23,24} Like asTF, flTF was also shown to enforce angiogenesis, chiefly via protease activated receptor (PAR)-

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2 activation through FVII and FX.²⁵

In the course of heart development, when cell proliferation and pro-angiogenic processes are intensified, asTF is expressed at increasingly higher levels compared to relatively constant levels of flTF and, in adult hearts, where developmental processes are complete, asTF and flTF exhibit similar levels and distribution patterns.¹⁷ These observations suggest that asTF may possibly play a role in developmental and/or regenerative processes, such as angiogenesis and cell proliferation. Here, we show that overexpression of murine asTF in murine cardiomyocytic HL-1 cells induces the expression of pro-angiogenic as well as pro-migratory factors. Moreover, the overexpression of murine asTF is associated with increased cell proliferation, migration of monocytic cells, and endothelial tube formation. These data indicate that murine asTF is a potential pro-migratory and pro-angiogenic factor in the myocardium. We propose that therapeutic induction of asTF in infarcted regions of the heart may enhance regenerative cell proliferation and angiogenesis, thereby improving the outcome of ischemic heart disease.

Methods

Cell Culture

Murine cardiomyocyte-like cells (HL-1) and human monocytic cells (THP-1) were cultured in Claycomb medium or RPMI medium (containing 10% fetal calf serum [FCS], PAA Laboratories, Pasching, Austria), respectively, at 37°C in a humidified incubator (5% CO₂, 95% air). Murine microvascular endothelial cells (MHEC5-T) were cultured in DMEM (containing 10% FCS, PAA Laboratories).

Overexpression Plasmids

For transfection experiments the plasmid pEGFP-N1 (Becton Dickinson, Heidelberg, Germany) was used. A cDNA construct of murine asTF was cloned into the multiple cloning site of the plasmid. The quality, orientation and identity of the asTF overexpression vector (pEGFP-N1_asTF) were confirmed by automated sequencing. The empty plasmid (pEGFP-N1_LV) was used for control transfections.

Stable Transfection of HL-1 Cells

A total of 5×10⁶ HL-1 cells per well were seeded in 6-well plates. Transfection of pEGFP-N1_asTF or pEGFP-N1_LV was done using Lipofectamin^{TM} 2000 (Invitrogen, Karlsruhe, Germany) following the manufacturer's protocol. Selection of stable transfectants was performed by incubating transfected cells with 800 µg/ml gentamycin (PAA Laboratories). Stable overexpression was continuously verified on quantitative reverse transcription–polymerase chain reaction (RT-PCR).

Real-Time PCR (TaqMan®)

Real-time PCR using specific gene expression assays for FGF2, Cyr61 and VEGF (Applied Biosystems, Carlsbad, CA, USA) was performed following the manufacturer's instructions.

Semi-Quantitative RT-PCR

Total RNA was reverse transcribed using AMV (Roche Diagnostics, Mannheim, Germany), and cDNAs encoding FGF2, Cyr61 and VEGF were amplified. Primers used for detection were as follows: FGF2_for, 5'-AGCGGCATC-ACCTCGCTTCC-3'; FGF2_rev, 5'-TGGAAGAAACAG-TATGGCCTTCTGTCC-3'; amplicon, 437 bp; Cyr61_for, 5'-GAAGGCAGACCCTGTGAATA-3'; Cyr61_rev, 5'-TG-

GTCTTGCTGCAT TTCTTG-3'; amplicon, 576 bp; VEGF_for, 5'-CTGCTCTCTTGGGTCCACTGG-3'; VEGF_rev, 5'-CA-CCGCTTGGCTTGTCACAT-3'; amplicons, 562 bp and 634 bp; β -actin_for, 5'-TGTACGTAGCCATCCAGGCT-3'; β -actin_rev, 5'-TTCTCCAGGGAGGAAGAGGA-3'; amplicon, 319 bp (Ocimum Biosolutions, Hyderabad, India). PCR conditions: 95°C, 2 min, and 36 cycles of 95°C, 30 s; 60°C, 25 s; and 72°C, 1 min.

Western Blotting

Western blot analysis of HL-1 lysates was performed as previously described.⁸ For protein detection, specific antibodies against FGF2 (Millipore, Schwalbach, Germany), Cyr61 (a kindly gift from Lester F. Lau, University of Illinois, Chicago, IL, USA), VEGF (Abnova, Heidelberg, Germany), phosphorylated as well as total ERK 1/2 and phosphorylated p38 (Cell Signalling Technology, Danvers, MA, USA), total p38 and GAPDH (Calbiochem, Darmstadt, Germany) were used.

In Vitro Cell Migration Assay

Transwell inserts with 8-µm pore size fitted in 24-well plates (Becton Dickinson) were used. A total of 1×10⁶ THP-1 cells were resuspended in 100 µl fresh RPMI without FCS. A total of 800 µl of the supernatant of asTF-overexpressing HL-1 cells or control HL-1 cells (transfected with empty plasmid) were placed into the lower chamber. FCS-free RPMI medium alone was used as a negative control. Cells were incubated for 48 h at 37°C. After that, cells that had migrated into the lower chamber were counted using a Neubauer counting chamber and a Leica DMIL light microscope (Leica, Wetzlar, Germany).

Recombinant Murine TF Proteins

flTF was obtained from American Diagnostica (Pfungstadt, Germany). Recombinant murine asTF mature peptide was produced in *Escherichia coli* and re-solubilized from bacterial inclusion bodies (a gift from the laboratory of Dr Vladimir Y. Bogdanov, University of Cincinnati, Cincinnati, OH, USA). For stimulation experiments different concentrations of recombinant (r)asTF or (r)flTF (0–1,000 nmol/L) were added to the FCS-free medium in the lower chamber to characterize the pro-migratory potential of these factors.

Tube Formation Assay

Angiogenesis Assay Kit (Millipore, Schwalbach, Germany) was used and experiments were performed following the manufacturer's protocol. In brief, 7.5×10³ MHEC5-T per well were resuspended in the supernatant of asTF-overexpressing HL-1 cells or cells transfected with empty plasmid. After that, these cells were seeded on 50 µl of the ECMatrix^{TM} in 96-well plates and incubated for 7 h at 37°C in humidified air with 5% CO₂. Tube formation by endothelial cells was analyzed using Leica DMIL light microscope (Leica).

Carboxyfluorescein Succinimidyl Ester Cell Proliferation Assays

HL-1 cells were labeled with carboxyfluorescein succinimidyl ester (CFSE, 5 µmol/L). After that, cells were washed and resuspended in Claycomb medium (containing 10% FCS; PAA Laboratories). A total of 1×10⁶ HL-1 cells per well were seeded in 12-well plates and incubated at 37°C in humidified air with 5% CO₂. Cell proliferation was measured after 24 h, 48 h and 72 h using flow cytometry at 488 nm.²⁶

Figure 1. Stable overexpression of alternatively spliced tissue factor (asTF) alters the expression pattern in HL-1 cells. (**A–C**) Analysis of the mRNA expression of (**A**) fibroblast growth factor-2 (FGF2), (**B**) cysteine-rich 61 (Cyr61) and (**C**) vascular endothelial growth factor (VEGF) in murine cardiomyocyte-like HL-1 cells normalized against GAPDH. Compared are HL-1 cells stably overexpressing asTF or full length TF (fITF), respectively, and control cells transfected with empty plasmid (control). (**A–C**) Real-time reverse transcription–polymerase chain reaction analyses given as mean $\pm$ SEM of at least 8 independent experiments. i.t., in trend $P < 0.15$; $^*P < 0.05$; and $^{\#}P < 0.01$. (**D**) Western blot analysis of intracellular protein expression of FGF2, Cyr61 and VEGF (representative of at least 3 independent experiments). GAPDH was used as loading control.

Ki67 Cell Proliferation Test

A total of 1×10^6 HL-1 cells were fixed in paraformaldehyde at 4°C overnight. After that, cells were centrifuged and resuspended in phosphate-buffered saline. Cells were permeabilized with 0.1% TritonX for 5 min, washed and centrifuged. Cells were incubated with an anti-Ki67 antibody for 1 h at room temperature. Thereafter, cells were washed and incubated with a second anti-mouse-M488 antibody for 45 min at room temperature. Finally, the cell proliferation was assessed using flow cytometry.²⁶

Statistical Analysis

All data are expressed as mean $\pm$ SEM. Data were analyzed using Student's t-test or 1-way ANOVA. $P \leq 0.05$ was deemed significant.

Results

Overexpression of the Soluble TF Isoform Induces Expression of Pro-Migratory and Pro-Angiogenic Factors in HL-1 Cells

Stable overexpression of murine asTF in murine cardiomyocyte-like HL-1 cells induced the expression of the pro-angiogenic and pro-migratory factors at the mRNA and protein levels (Figure 1). Compared to control cells transfected with empty plasmid or cells overexpressing fITF, the mRNA ex-

pression of FGF2 (Figure 1A) and Cyr61 (Figure 1B) was significantly increased in asTF-overexpressing HL-1 cells. Moreover, asTF overexpression also induced the mRNA expression of VEGF in these cells compared to control cells and fITF-overexpressing HL-1 cells (Figure 1C). On the protein level, the expression of FGF2, Cyr61, and VEGF was appreciably increased in asTF-overexpressing cardiomyocyte-like HL-1 cells (Figure 1D).

Overexpression of asTF Increases Proliferation of HL-1 Cells

Stable overexpression of asTF in HL-1 cells significantly increased cell proliferation, compared to control cells transfected with empty plasmid (Figures 2A,B). In contrast to stable overexpression, siRNA-mediated downregulation of total TF in HL-1 cells led to a significant reduction of cell proliferation compared to control cells (Figures 2A,B). The inhibitory impact of siRNA on total TF expression was verified on western blotting and semi-quantitative RT-PCR (data not shown). Furthermore, the stimulation of HL-1 cells with (r)asTF induced cell proliferation in a dose-dependent manner (Figure 2C). In contrast to (r)asTF, (r)fITF had no significant impact on cell proliferation (Figure 2D). Treatment of HL-1 cells with lipopolysaccharide (LPS) at equimolar concentrations did not cause the increase in cell proliferation observed with (r)asTF (data not shown).

Figure 2. Overexpression of murine alternatively spliced tissue factor (asTF) increases proliferation of murine cardiomyocytic HL-1 cells. Shown is impact of murine asTF overexpression on cell proliferation as (A) the relative proliferation change or (B) increase in fluorescence intensity, respectively. Compared are asTF-overexpressing HL-1 cells (asTF), cells pre-treated with inhibitory siRNAs against total TF (siTF), and HL-1 cells stably transfected with empty plasmid (control). (C,D) Characterization of the influence of (C) recombinant (r)asTF or (D) (r)full length TF [(r)flTF], respectively, on cell proliferation. To test the possible influence of endotoxin contamination, different concentrations of lipopolysaccharide (LPS, 0.1–100 nmol/L) were used. Compared are wild-type HL-1 cardiomyocytic cells treated for 48 h with different concentrations of (r)asTF or (r)flTF ranging from 0 to 1,000 nmol/L. The mean $\pm$ SEM of 5–8 independent experiments is shown. i.t., in trend $P < 0.15$; * $P < 0.05$; # $P < 0.01$; § $P < 0.001$.

Impact of asTF Overexpression on THP-1 Migration

The pro-migratory effect of asTF overexpression on the chemotaxis of monocytic THP-1 cells was analyzed using a Boyden migration chamber. The supernatants of asTF-overexpressing HL-1 cells and control cells were analyzed with regard to the pro-migratory potential. The supernatant of asTF-overexpressing HL-1 cells significantly increased the migration of THP-1 cells, compared to the supernatant of HL-1 cells transfected with empty plasmid (Figure 3A). Treatment with (r)asTF induced the migration of THP-1 cells in a dose-dependent manner (Figure 3B). A significant induction of chemotaxis was achieved when stimulating the cells with 1 nmol/L up to 1,000 nmol/L (r)asTF. In contrast to (r)asTF, (r)flTF had no significant influence on THP-1 migration (Figure 3C). Treatment of cells with LPS at equimolar concentrations did not cause the increase in THP-1 migration observed with (r)asTF (Figures 3B,C).

Supernatant of asTF-Overexpressing HL-1 Cells Increases Endothelial Tube Formation

To evaluate the possible pro-angiogenic effect of asTF overexpression in HL-1 cells, the impact of the supernatant of

these cells on tube formation by murine endothelial cells was assessed (Figure 4). The supernatant of stable transfected asTF-overexpressing HL-1 cells significantly increased the tube formation activity of murine endothelial MHEC5-T cells compared to the supernatant of control cells, indicating that asTF may potentiate pro-angiogenic potential of HL-1 cells (Figure 4).

Possible Regulatory Pathways Involved in the asTF-Induced Effects on the Expression of Chemotaxis- and Angiogenesis-Promoting Factors in HL-1 Cells

In order to delineate the regulatory pathways underlying the observed asTF-associated effects on HL-1 cells, the phosphorylation states of the signaling molecules involved in the regulation of VEGF, FGF2, and Cyr61 expression were examined. Stimulation of HL-1 cells with 100 nmol/L (r)asTF reduced the phosphorylation of ERK 1/2 but increased the phosphorylation of p38 as compared to non-treated control cells (Figure 5A). In cells overexpressing asTF the phosphorylation pattern of ERK 1/2 and p38 was modified in the same manner as seen after stimulation with (r)asTF. No impact of (r)asTF or asTF overexpression in HL-1 cells on the phos-

Figure 3. The supernatant of alternatively spliced tissue factor (asTF)-overexpressing HL-1 cells increases the migration of monocytic THP-1 cells. **(A)** Fold induction of chemotaxis of THP-1 cells treated with the supernatant of asTF-overexpressing cardiomyocytic cells (asTF), or HL-1 cells stably transfected with empty plasmid (control), respectively. **(B, C)** Impact of different concentrations of **(B)** recombinant (r)asTF or **(C)** (r)full length TF [(r)flTF] ranging from 0 to 1,000 nmol/L on the number of migrated THP-1 cells after 48 h. To test the impact of endotoxin contamination, different concentrations of lipopolysaccharide (LPS, 0.1–100 nmol/L) were used. Mean±SEM of at least 3 independent experiments. i.t., in trend $P<0.15$; $^*P<0.05$; $^{\#}P<0.01$; $^{\$}P<0.001$.

phorylation state of JNK was detectable (data not shown). Moreover, the inhibition of ERK 1/2 and p38 differentially modified the mRNA expression pattern of VEGF, FGF2 and Cyr61 in non-treated wild-type HL-1 cells, as well as wild-type HL-1 cells stimulated with 100 nmol/L (r)asTF (**Figure 5B**).

Discussion

In the present study we report for the first time that overexpression of asTF in the murine cardiomyocyte-like cell line HL-1 increases the expression of pro-migratory as well as pro-angiogenic factors FGF2, Cyr61 and VEGF. Furthermore, we show that asTF overexpression increases cell proliferation and pro-angiogenic properties of HL-1 cells.

asTF Overexpression Increases Proliferation of HL-1 Cells

In the present study we found the overexpression of murine asTF to be associated with increased proliferation of murine cardiomyocyte-like HL-1 cells. These results are in line with the data of Hobbs et al, who reported that human asTF overexpression in pancreatic cancer cells increases tumor cell proliferation.²³ Moreover, overexpression of murine asTF in HL-1 cells was associated with an increased expression of FGF2, Cyr61 and VEGF. All these factors are known to increase cell proliferation.^{3–6}

We therefore hypothesize that the increase in cell prolifer-

Figure 4. Impact of the supernatant from alternatively spliced tissue factor (asTF)-overexpressing HL-1 cells on endothelial tube formation. Fold induction of tube formation of MHEC5-T cells treated with the supernatant of asTF-overexpressing HL-1 cells (asTF), or cells stably transfected with empty control plasmid (control), respectively. Mean±SEM of at least 3 independent experiments. $^{\$}P<0.001$.

Figure 5. Effect of murine alternatively spliced tissue factor (asTF) on regulatory signaling pathways in HL-1 cells. **(A)** Impact of recombinant (r)asTF or stable overexpression of asTF in HL-1 cells, respectively, on **(Left)** the phosphorylation state of ERK 1/2 and p38 and on **(Right)** total ERK 1/2 and total p38, respectively. Stimulation was carried out with 100 nmol/L (r)asTF, and compared to asTF-overexpressing HL-1 cells (asTF). Non-treated wild-type cells were used as controls (control). **(B)** Impact of pharmacologic inhibition of ERK 1/2 with 10 μ mol/L PD98059 and of p38 with 10 μ mol/L SB203580 on the mRNA expression of vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF2) and cysteine-rich 61 (Cyr61) in wild-type (wt) HL-1 cells, 2 h after stimulation of cells with 100 nmol/L (r)asTF. **(C)** Representative western blot analysis of the influence of pharmacologic inhibitors PD98059 (10 μ mol/L) or SB203580 (10 μ mol/L), respectively, on the phosphorylation of ERK 1/2 and p38.

eration associated with the overexpression of asTF in cardiomyocytic HL-1 cells is, at least in part, mediated by an asTF-induced enhancement of the expression of the proliferation-promoting factors FGF2, Cyr61 and VEGF. This hypothesis was substantiated by results of western blot analyses and RT-PCR. ERK 1/2 as well as p38 are known to be involved in the regulation of expression of FGF2, Cyr61 and VEGF.²⁷ These signaling factors are, among others, downstream effectors of PAR-1 and PAR-2.^{27,28} Our data showed that the stimulation of HL-1 cells with (r)asTF as well as the stable asTF overexpression in these cells, modified the phosphorylation state of ERK 1/2 and p38. This is in line with the results of van den Berg et al showing (r)asTF to induce the phosphorylation of p38 in human endothelial cells.²⁴ Blocking of ERK 1/2 as well as p38 altered the expression pattern of FGF2, Cyr61 and VEGF in non-stimulated and (r)asTF-stimulated HL-1 cells. Thus, the asTF-induced impact on the expression of the pro-migratory factors FGF2, Cyr61 and VEGF seems to be mediated via ERK 1/2 and p38 phosphorylation, although it cannot be excluded that additional signaling cascades may also play a role.

Membrane-bound fTTF complexes and activates FVII/

FVIIa, thereby initiating the blood coagulation cascade.^{29–31} The fTTF:FVIIa complex was demonstrated to induce the expression of Cyr61 as well as VEGF.^{32,33} Moreover, this complex was also suggested to increase the expression of FGF2 indirectly via activation of PAR signaling.³⁴ Bogdanov et al showed that the sequence involved in FVIIa binding is maintained in human and murine asTF.^{14,17} It is therefore probable that asTF may be able to bind and activate FVII/FVIIa, leading to associated downstream signaling events.^{14,30,35} In our experiments we used medium containing FCS. Neither data from the manufacturer nor western blot analyses showed FVII to be present in the culture medium or the FCS. But we cannot exclude the possibility that FVII was involved in the asTF-induced effects on HL-1 cells. Therefore, we analyzed whether recombinant (r)asTF or (r)fTTF induce HL-1 cell proliferation in medium without FCS. The results clearly show that (r)asTF but not (r)fTTF induces the cell proliferation of cardiomyocytic cells in a dose-dependent manner. Van den Berg et al found intratumoral human asTF protein concentrations to be in the range of 25–75 nmol/L.²⁴ Moreover, they showed highly pure and endotoxin-free human recombinant asTF to induce cell migration of human endothelial cells

already at a concentration of 1 nmol/L. In line with these observations, we found murine (r)asTF to stimulate cell proliferation of cardiomyocyte-like HL-1 cells at 1 nmol/L, which is likely to comprise a pharmacologically relevant concentration. Thus, asTF may directly or indirectly induce the expression of FGF2, Cyr61 and VEGF in murine asTF-overexpressing HL-1 cells, thereby contributing to increased cell proliferation.

Overexpression of asTF in HL-1 Cells Induces Migration of Monocytic Cells

Herein it was shown that the supernatant of murine asTF-overexpressing cardiomyocyte-like HL-1 cells promotes the chemotaxis of THP-1 cells. In line with our data, van den Berg et al found that human asTF induces migration of human endothelial cells via integrin signaling.²⁴ In this study, no direct interaction of asTF with integrins could be demonstrated. Therefore, it is possible that the migration-promoting effect of the soluble murine TF isoform was not directly mediated via asTF-integrin interactions. Further studies are needed to investigate whether murine asTF ligates integrins, similar to human asTF.²⁴

The supernatant of asTF-overexpressing HL-1 cells increases the chemotaxis of THP-1 cells, and asTF overexpression is associated with an increased expression of the secreted and soluble pro-migratory factors FGF2, Cyr61 and VEGF. The pro-migratory effect of FGF2, Cyr61, as well as VEGF, was previously demonstrated.^{3–6,32,36} As aforementioned, we tested whether different concentrations of (r)asTF or (r)flTF also induce THP-1 chemotaxis. We found (r)asTF but not (r)flTF to induce cell migration in a dose-dependent manner, even at low, more physiologic, concentrations (1 nmol/L). These observations are in line with the data of van den Berg et al, which demonstrated (r)asTF to induce the migration of human endothelial cells at 1 nmol/L.²⁴ Moreover, from the results of our migration experiments we can estimate that the intensity of chemoattraction achieved by stable asTF overexpression is comparable with the induction of cell migration achieved with 10–50 nmol/L (r)asTF. There is little information available regarding the physiologic or pathophysiologic levels of asTF in human or murine organisms. Van den Berg et al showed 25–75 nmol/L to be a pathophysiologically relevant intratumoral concentration range of asTF in patients suffering from cervical cancer.²⁴ Therefore, the concentration reached by asTF overexpression in the present experiments is likely to be within this, pathophysiologically relevant, range. In sum, our data suggest that murine asTF is pro-migratory, and that this property of asTF is possibly mediated by increasing the expression of the pro-migratory factors FGF2, Cyr61 and VEGF.

asTF Overexpression in HL-1 Cells Potentiates Endothelial Tube Formation

Recently, van den Berg et al showed that human asTF induces tube formation by human endothelial cells in vitro as well as sprouting in vivo.²⁴ Here, the supernatant of murine asTF-overexpressing cardiomyocytic HL-1 cells was found to induce the tube formation by murine microvascular endothelial cells. These observations are in full agreement with the results of van den Berg et al.²⁴

As mentioned earlier, asTF overexpression in HL-1 cells was associated with an increased expression of FGF2, Cyr61 as well as VEGF. All these factors are known to be very pro-angiogenic.^{3–6,37,38} Therefore, it is probable that the stimulation of endothelial tube formation by the supernatant of

asTF-overexpressing HL-1 cells is due to the increased expression of FGF2, Cyr61 and VEGF.

In summary, the present results indicate that the induction of cell proliferation, monocyte chemotaxis, and endothelial tube formation – events elicited by the overexpression of murine asTF in cardiomyocyte-like HL-1 cells – are likely mediated by an increased expression of FGF2, Cyr61 and VEGF. These processes appear to be mediated by signaling cascades involving ERK 1/2 and p38. Thus, we propose that asTF is a potential chemotaxis- and angiogenesis-promoting factor that participates in developmental and regenerative processes, such as angiogenesis.

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