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p27^{Kip1} inhibits tissue factor expression

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ABSTRACT

Background: The cyclin-dependent kinase inhibitor (CDKI) p27^{Kip1} regulates cell proliferation and thus inhibits atherosclerosis and vascular remodeling. Expression of tissue factor (TF), the key initator of the coagulation cascade, is associated with atherosclerosis. Yet, it has not been studied whether p27^{Kip1} influences the expression of TF.

Methods and results: p27^{Kip1} overexpression in human aortic endothelial cells was achieved by adenoviral transfection. Cells were rendered quiescent for 24 h in 0.5% fetal-calf serum. After stimulation with TNF-α (5 ng/ml), TF protein expression and activity was significantly reduced ($n = 4$; $P < 0.001$) in cells transfected with p27^{Kip1}. In line with this, p27^{Kip1} overexpression reduced cytokine-induced TF mRNA expression ($n = 4$; $P < 0.01$) and TF promotor activity ($n = 4$; $P < 0.05$). In contrast, activation of the MAP kinases p38, ERK and JNK was not affected by p27^{Kip1} overexpression.

Conclusion: This in vitro study suggests that p27^{Kip1} inhibits TF expression at the transcriptional level. These data indicate an interaction between p27^{Kip1} and TF in important pathological alterations such as atherosclerosis and vascular remodeling.

1. Introduction

Atherosclerosis represents a major public health burden. Percutaneous coronary intervention is the preferred treatment option for patients presenting with an acute coronary syndrome [1]. Nevertheless, delayed reendothelialisation [2] and stent thrombosis [3,4] still represent a concern leading to reintervention in some patients.

Tissue factor, a 47-kDa transmembrane protein, is the key initiator of coagulation and thrombosis [5,6]. By binding to FVII or FVIIa, the coagulation cascade is initiated, resulting in thrombin generation and, finally, thrombus formation. Apart from its well-known role in hemostasis and thrombosis, the TF-FVIIa bimolecular complex acts as a cellular receptor and exerts a broad range of intracellular signalling events [7]. These pathways are regulated either by TF itself via its cytoplasmatic domain or, alternatively, through interaction with protease-activated receptors (PARs) on the cell surface. Via upregulation of vascular endothelial growth factor (VEGF) [8], interleukin-8 (IL-8) [9], and connective tissue growth factor [10], TF may enhance migration and proliferation of vascular smooth muscle and endothelial cells [11], thereby modulating vascular remodeling and reendothelialization. In the inflammatory environment of the vessel wall after percutaneous intervention and in different stages of atherosclerosis, TF is upregulated [12,13]; nevertheless, the mechanisms responsible for the increased TF expression have not been fully explored.

Progression through the cell cycle is regulated by specific cyclin–cyclin dependent kinase complexes [14]. By controlling the entry of the cell into the next phase of the cell cycle, these complexes regulate cell proliferation, whereas their activity is inhibited by cyclin-dependent kinase inhibitors (CKIs) [15]. Two families of CKIs have been identified so far: the Kip/Cip proteins (p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}), and the INK proteins (p15^{INKB}, p16^{INKA}, p18^{INKC}, and p19^{INKD}) [16]. Recent studies have established that the Kip/Cip proteins do not only act as inhibitors of cell cycle progression, but may also exert additional functions [14]. p21^{Cip1} interacts directly with Myc [17] and STAT3 [18], and p27^{Kip1} downregulates JAB1, a co-factor of the transcription factor AP-1 [19]. These results indicate that the CKIs may also influence gene transcription.

Expression of p27^{Kip1} is downregulated after balloon injury resulting in accelerated cell proliferation. Since downstream targets of p27^{Kip1} are important transcription factors regulating the

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expression of TF, the question arises whether $p27^{Kip1}$ may regulate the expression of TF.

2. Materials and methods

2.1. Cell culture and adenoviral infection

Human aortic endothelial cells (HAECs; Clonetics, Allschwil, Switzerland) were cultured as described [20]. Briefly, adhering HAECs were grown to confluence and rendered quiescent for 24 h in medium containing 0.5% FCS before stimulation. Cells were stimulated with 5 or 10 ng/ml tumor necrosis factor alpha (TNF-α; R&D Systems, Minneapolis, MN). Recombinant, replication-defective, E1A/E3-deleted adenoviral vectors encoding human $p27^{Kip1}$ and a control vector, AdE1, were constructed with an expression cassette containing a cytomegalovirus promoter and bovine growth human polyadenylation signal. Adenoviral transduction of HAECs was performed at a multiplicity of infection of 100. Cytotoxicity was assessed by a colorimetric assay to detect lactate dehydrogenase (LDH; Roche, Basel, Switzerland).

2.2. Western blot analysis

Protein expression was determined as described [20]. Antibodies against human TF (American Diagnostica, Stamford, CT) and vascular cell adhesion molecule-1 (VCAM-1; Santa Cruz Biotechnology, Santa Cruz, CA) were both used at 1:2000 dilution. Antibodies against phosphorylated p38 mitogen-activated protein (MAP) kinase (p38), p44/42 MAP kinase (ERK), and c-Jun NH2-terminal kinase (JNK; all from Cell Signaling, Danvers, MA) were used at 1:1000, 1:5000, and 1:1000 dilution, respectively. Antibodies against total p38, ERK, and JNK (all from Cell Signaling) were diluted to 1:3000, 1:2000, and 1:1000, respectively. Alpha-tubulin (aT; Sigma) was applied to control for protein loading (1:10000 dilution). Primary antibodies were detected with a horseradish peroxidase-linked secondary antibody (Amersham, Munich, Germany).

2.3. Real-time PCR

Total RNA was extracted from HAECs with 1 mL TRIzol Reagent (Invitrogen, Lucerne, Switzerland) as described [20]. Conversion of total cellular RNA to cDNA was carried out with Moloney murine leukemia virus reverse transcriptase and random hexamer primers (Amersham) in a final volume of 33 μL using 4 μg of RNA. The total cDNA pool obtained served as template for subsequent PCR amplification with primers specific for full-length TF (sense primer: 5'-TCCCCAGAGTTCACACCTTACC–3', antisense primer: 5'-CCTTTCTCCTGGCCCATACAC–3'; bases 508–529 of *F3* cDNA; NCBI No. NM 001993). Real-time PCR amplification was performed in an MX3000P PCR cycler (Stratagene) using the SYBR Green JumpStart kit (Sigma) in 25 μL final reaction volume containing 2 μL cDNA, 10 pmol of each primer, 0.25 μL of internal reference dye, and 12.5 μL of JumpStart Taq ReadyMix (buffer, dNTP, stabilizers, SYBR Green, Taq polymerase, and JumpStart Taq antibody). A melting curve analysis was performed after amplification to verify the accuracy of the amplicon. Ribosomal L28 RNA served as loading control.

2.4. TF activity

Whole TF activity in HAECs was analyzed using a colorimetric assay (American Diagnostica). Cell lysates were incubated at 37 °C with human FVIIa and FX, allowing for the formation of TF/FVIIa complex. Conversion of FX to FXa was measured by the ability of FXa to cleave a chromogenic substrate. A standard curve was established with lipidated human TF to assure that the results were in the linear range of detection.

2.5. TF promoter activity

An adenoviral vector (Ad5/hTF/Luc) containing the minimal TF promoter (−227 bp to +121 bp) upstream of the Luciferase cDNA and the SV40 PolyA signal was prepared as described [21]. For viral transfection, the vector was added to HAEC at 100 pfu/cell for 1 h. HAEC were kept in growth medium for 24 h and then serum starved for 24 h prior to TNF- stimulation. Cells were stimulated with TNF-α for 30 min. Firefly luciferase activity was determined in cell lysates using a luminometer (Bertholg Technologies, Bad Wildbad, Germany).

2.6. Cell cycle analysis

Cell cycle distribution was analyzed through DNA content by flow cytometry using propidium iodide. After a 24 h quiescence period, cells were collected and fixed in 100% ethanol. After one hour, cells were permeabilized in 0.1% Triton-X for 15 min and stained with propidium iodide for FACS analysis.

2.7. Statistical analysis

Data are indicated as mean ± SEM. Unpaired Student's *t*-test was used to evaluate differences between two groups. For statistical analysis, one-way ANOVA was performed with a post hoc analysis. A P value <0.05 denoted a significant difference.

3. Results

3.1. $p27^{Kip1}$ overexpression inhibits endothelial cell proliferation

Successful transfection of $p27^{Kip1}$ was confirmed by Western blot analysis (Fig. 1A). Overexpression of $p27^{Kip1}$ significantly inhibited serum-stimulated proliferation of HAECs ($n = 3$; $P < 0.001$; Fig. 1B) after 48 h. No changes in cell morphology were detected, while there was a tendency to reduced LDH release in $p27^{Kip1}$ transfected cells ($n = 4$; P = NS; data not shown).

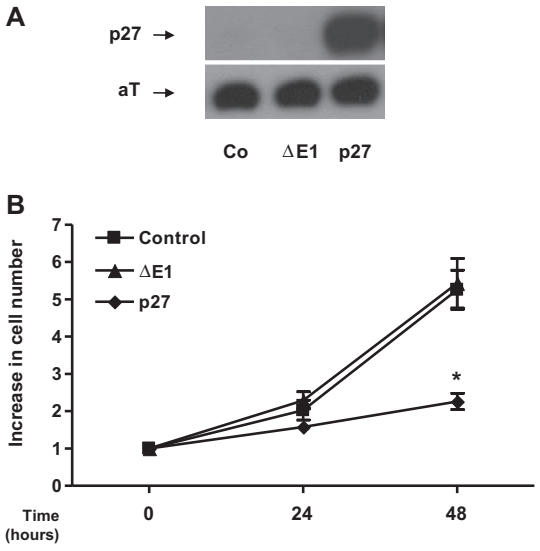

Fig. 1. Overexpression of $p27^{Kip1}$ inhibits endothelial cell proliferation. (A) $p27^{Kip1}$ adenoviral transfection of human aortic endothelial cells (HAECs). (B) Overexpressed $p27^{Kip1}$ protein is physiologically active as shown by inhibition of cell proliferation (*$P < 0.001$).

3.2. $p27^{Kip1}$ inhibits cytokine-induced TF protein expression and activity

TF protein expression was determined in TNF-α (5 ng/mL for 5 h) stimulated HAECs with or without $p27^{Kip1}$ overexpression, respectively. TNF-α induced a significant increase in TF protein expression compared to control conditions, and $p27^{Kip1}$ overexpression diminished this effect ($n = 4$; $P < 0.001$; Fig. 2A). The changes in TF protein level were paralleled by a decrease in TF activity ($n = 3$; $P < 0.001$; Fig. 2B). In contrast, basal TF levels ($n = 4$; P = NS; Fig. 2C) were not affected by $p27^{Kip1}$ overexpression. Expression of vascular cell adhesion molecule-1 (VCAM-1) was not affected by $p27^{Kip1}$ transfection ($n = 4$; P = NS; Fig. 2D).

3.3. $p27^{Kip1}$ overexpression does not alter cell cycle phase of growth-arrested cells

Experiments were performed in cells rendered quiescent in 0.5% FCS for 24 h. To determine the phase of the cell cycle in which HAECs were arrested, propidium iodide staining was performed and the cells analyzed by flow cytometry. As shown in supplemental figure 1, rendering the cells quiescent for 24 h in 0.5% FCS promoted a G_0/G_1-arrest. This effect did not differ in $p27^{Kip1}$ transfected cells and controls ($n = 4$; P = NS; Supplemental Fig. 1).

3.4. $p27^{Kip1}$ inhibits TF mRNA expression

Real-time rtPCR revealed that TNF-α (5 ng/mL) significantly induced TF mRNA expression ($n = 4$; $P < 0.01$; Fig. 3A). $p27^{Kip1}$ overexpression inhibited this effect ($n = 4$; $P < 0.01$; Fig. 3A).

3.5. $p27^{Kip1}$ inhibits TF promoter activity

To assess whether $p27^{Kip1}$ inhibits TF mRNA expression by reducing TF promoter activity, the impact of $p27^{Kip1}$ overexpression on TF promoter activity was analyzed. HAECs were transfected with a luciferase expression plasmid under control of the human minimal TF promoter (−221 bp up to +121 bp) followed by transfection with the $p27^{Kip1}$ encoding plasmid. TNF-α induced a significant increase in TF promoter activity ($n = 5$; $P < 0.01$; Fig. 3B). $p27^{Kip1}$ overexpression inhibited this effect ($n = 5$; $P < 0.05$; Fig. 3B).

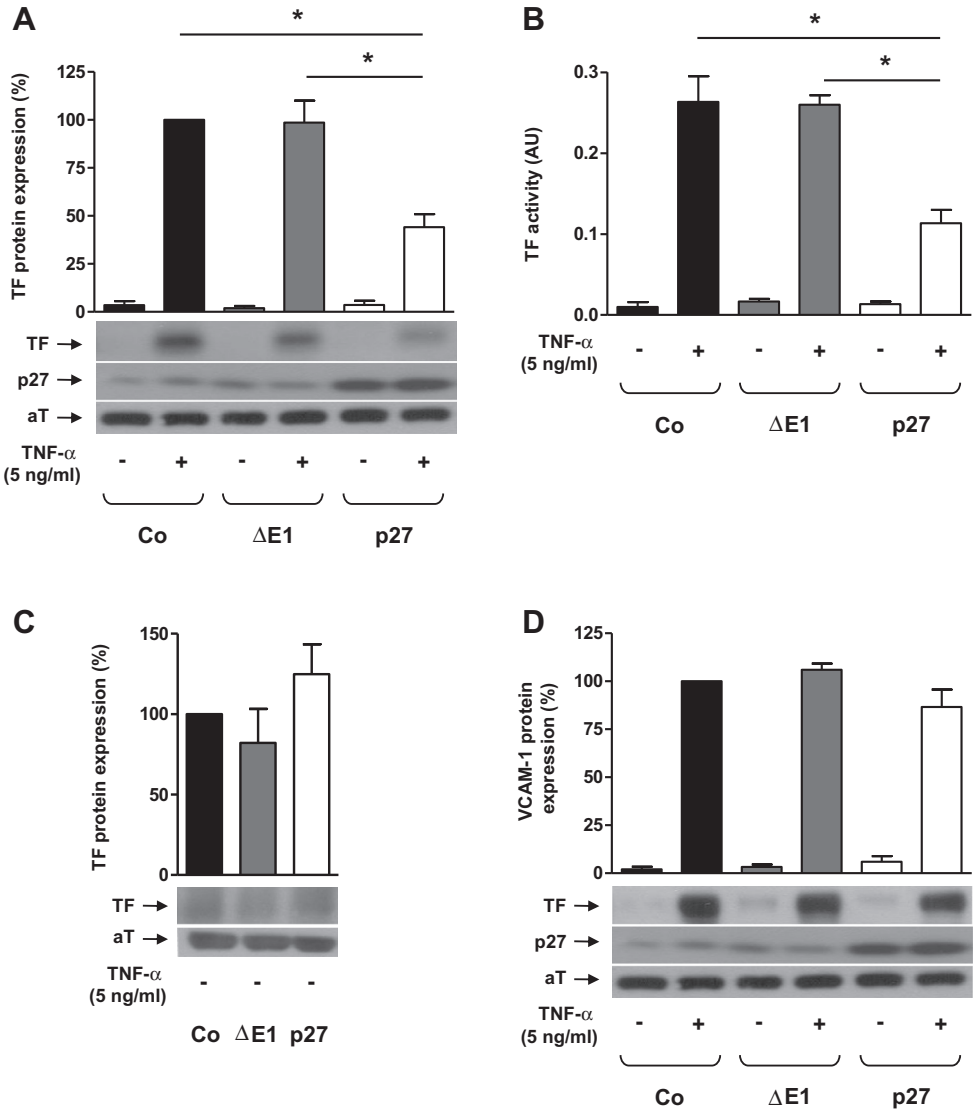

Fig. 2. $p27^{Kip1}$ overexpression inhibits TNF-α induced TF protein expression and activity. A and B TF protein expression and activity is reduced by $p27^{Kip1}$ overexpression in HAECs (*$P < 0.001$). C and D. Basal TF and TNF-α induced VCAM-1 protein expression are not changed by $p27^{Kip1}$ transfection (P = NS).

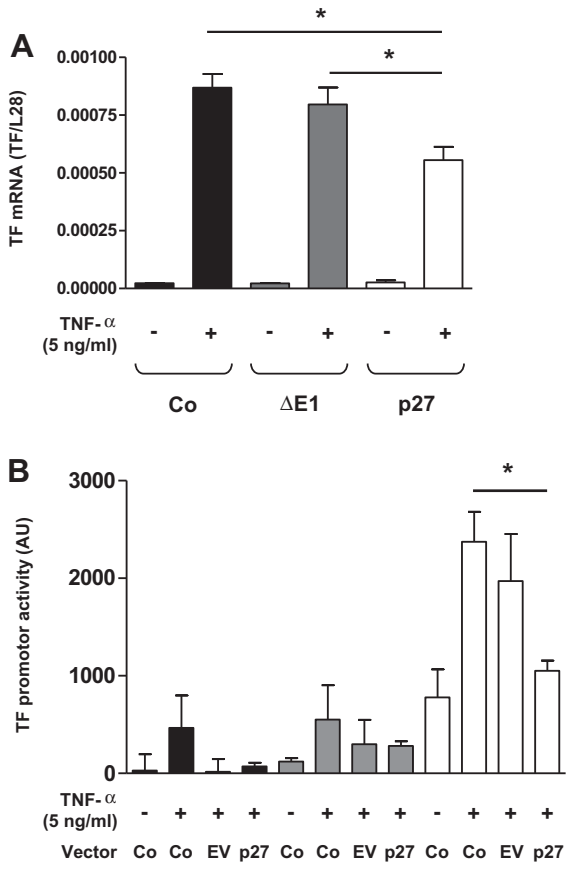

Fig. 3. TF mRNA expression and TF promotor activity are reduced by $p27^{Kip1}$. (A) Real-time PCR reveals that $p27^{Kip1}$ transfection inhibits TNF-α induced TF mRNA expression (*$P < 0.01$ vs TNF-α alone). Values are indicated as percent of TNF-α alone and normalized to L28 expression. (B) TNF-α induced TF promotor activity is reduced in cells transfected with $p27^{Kip1}$ (*$P < 0.05$).

3.6. $p27^{Kip1}$ overexpression does not affect MAP kinase activation

To assess whether $p27^{Kip1}$ alters MAP kinase activation, HAECs were examined at different time points after TNF-α stimulation. The MAP kinases p38, ERK, and JNK were transiently activated by TNF-α ($n = 3$; Fig. 4). Phosphorylation of p38, ERK, and JNK remained unaffected by $p27^{Kip1}$ overexpression ($n = 3$; P = NS; Fig. 4).

4. Discussion

This study demonstrates that $p27^{Kip1}$ reduces the expression of TF in endothelial cells in vitro by inhibiting the activity of the TF promotor.$p27^{Kip1}$ is a cell-cycle regulator inhibiting cell proliferation by inducing cell cycle arrest in G1 phase, while it is downregulated during the other phases of the cell-cycle. In highly proliferating cells such as arterial endothelial and smooth muscle cells after balloon injury, $p27^{Kip1}$ expression is diminished markedly thereby permiting cell proliferation [16]. This is in contrast to the behaviour of TF; expression of the latter is downregulated in cells at rest and increases in highly proliferating cells [13]. The present study demonstrates for the first time not only an inverse expression pattern of $p27^{Kip1}$ and TF, but also a direct regulatory link between the two proteins.

In addition to their well-known role in cell cycle regulation, there is growing evidence that CKIs also act as regulators of gene expression [14,19]. $p27^{Kip1}$ regulates proliferation and migration of endothelial and vascular smooth muscle cells which contribute

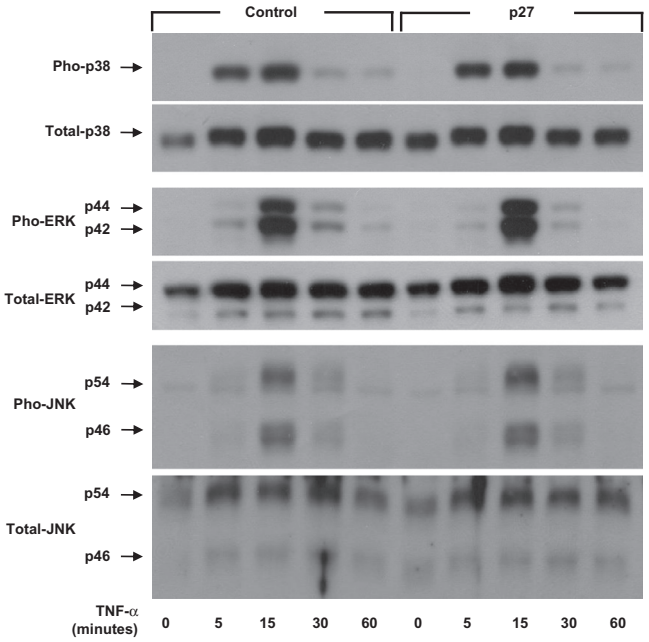

Fig. 4. MAP kinase activation is not altered by $p27^{Kip1}$. The MAP kinases JNK, p38, and ERK are transiently activated by TNF-α (5 ng/ml). Phosphorylation of JNK, p38 and ERK are not affected by $p27^{Kip1}$. No significant change in total expression of MAP kinases is observed (P = NS).

to atherosclerosis, restenosis, and reendothelialisation after coronary intervention [16,22–24]. In an animal model, a correlation between $p27^{Kip1}$ expression and the development of atherosclerosis was established in apolipoprotein E (apoE)-null mice [25], since deletion of $p27^{Kip1}$ in these mice induced an accelerated atherogenesis. In humans, an inverse correlation between $p27^{Kip1}$ expression and the level of atherosclerosis was observed [16]. On the other hand, TF is crucially involved in these processes [26–28] as well, and the level of TF increases with the stage of atherosclerosis [29]. The inverse expression pattern of $p27^{Kip1}$ and TF in atherosclerotic lesions is in line with our finding that $p27^{Kip1}$ regulates the expression of TF.

Upregulation of cellular $p27^{Kip1}$ inhibits TF expression at the mRNA level. This novel observation does not result from the cell-cycle regulatory effect of $p27^{Kip1}$, since experiments were performed in cells rendered quiescent for 24 h in medium containing 0.5% FCS which promotes a G_0/G_1-arrest in all the experimental groups independent of whether $p27^{Kip1}$ was overexpressed. This interpretation was confirmed by propidium iodide staining, and this finding is in line with increasing evidence that CKIs act as transcriptional regulators independent of their cell-cycle regulatory function [14]. $p27^{Kip1}$ downregulates Jun activating binding protein 1 (JAB1), a cofactor of the transcription factor AP-1, and thereby interacts with protein expression [19]. Further, in a human hepatocellular cancer cell line, overexpression of $p27^{Kip1}$ downregulates the level of IB- which is necessary for the activation of NFB [30]. Our data therefore support the growing evidence on a direct regulatory function of CKIs on protein expression.

Similar to the development of atherosclerosis, restenosis after balloon angioplasty and stent implantation is a well-known clinical problem. After intervention, $p27^{Kip1}$ is downregulated at the site of the injury thereby permitting vascular repair through enhanced endothelial and vascular smooth muscle cell proliferation and migration [16]. This process leads to rapid reendothelialisation and repair, but may also result in restenosis under pathological conditions. Local delivery of an adenovirus encoding for $p27^{Kip1}$ significantly attenuates restenosis [23], and in patients with a

single nucleotide polymorphism in the $p27^{Kip1}$ gene presents with a higher level of restenosis [31]. Constistent with these observations and similar to the development of atherosclerotic lesions, increasing levels of TF have been shown to be associated with restenosis [32,33]. Again, $p27^{Kip1}$ and TF are inversely correlated at the site of restenosis supporting our observation that $p27^{Kip1}$ regulates TF expression.

In summary, the present in vitro study demonstrates that $p27^{Kip1}$ inhibits TF expression at the transcriptional level. These findings add further information on the molecular processes involved in pathological diseases such as atherosclerosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.002.

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