Density-Dependent Shift of Transforming Growth Factor-Beta-1 from Inhibition to Stimulation of Vascular Smooth Muscle Cell Growth Is Based on Unconventional Regulation of Proliferation, Apoptosis and Contact Inhibition

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Key Words

Apoptosis · Contact inhibition · Proliferation · Transforming growth factor- β · Vascular smooth muscle cell

Abstract

Background: TGF- β shifts from inhibition to stimulation of vascular smooth muscle cell (vSMC) growth when cell density increases. How proliferation and apoptosis contribute to this shift is still unknown. **Methods:** In sparse and confluent V8 vSMC treated or not with TGF- β_1 (1 ng/ml) for 3 days, cell number, mitotic activity, cell-cycle-regulatory protein levels, caspase-3 and phosphoinositide 3-kinase (PI3-K) activities were studied. **Results:** In TGF- β_1 -treated cells, (i) the growth curve rose constantly compared to controls, reaching post-confluent densities; (ii) mitotic activity, which was constant at all cell densities, was lower than in sparse but higher than in contact-inhibited control cells, and (iii) apoptosis occurred at sparse densities only. The mechanism of proliferation control by TGF- β_1 was very unconventional in V8 vSMCs: (i) p15^{INK4b} and cyclin D levels were similar in cells treated or not with TGF- β_1 , and (ii) p27^{Kip1} levels remained very low even at high densities while cyclin E levels were not markedly decreased. TGF- β_1 -induced apoptosis in sparse cultures and its reversal in dense cultures were inversely correlated to PI3-K

activation. **Conclusions:** TGF- β_1 slowed sparse V8 vSMC growth by inhibiting proliferation and inducing apoptosis. TGF- β_1 -treated confluent vSMCs escaped contact inhibition and kept growing through unconventional regulation of p27^{Kip1}, cyclin E and suppression of apoptosis.

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Introduction

In mature blood vessels, normal vascular smooth muscle cells (vSMCs) are arrested at the G₀/G₁ boundary of the cell cycle and enter the mitotic phase at very low frequency [1]. However, quiescent vSMCs are capable of re-entering and progressing through the cell cycle under pathological situations like arteriosclerosis, hypertension and restenosis following vascular graft or injury [2–6]. The proliferation of vSMCs is controlled by many growth factors among which TGF- β has received special attention.

Progression through the G₁ phase of the cell cycle requires the activation of specific complexes between cy-

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clins and cyclin-dependent kinases (CDKs), e.g. cyclin D/CDK4, cyclin D/CDK6 and cyclin E/CDK2. The activity of the CDKs is also regulated by CDK inhibitors (CKIs) associated to the cyclin/CDK complexes. The CKIs belong to two families. The Kip-Cip CKIs (p21^{Cip1}, p27^{Kip1} and p57^{Kip2}) inhibit cyclin E/CDK2 complexes but bind to and act as positive regulators of the cyclin D/CDK4 and cyclin D/CDK6 complexes. The INK CKIs (p15^{INK4b}, p16^{INK4a}, p18^{INK4c} and p19^{INK4d}) exclusively bind and inhibit cyclin D/CDK4/Kip-Cip and cyclin D/CDK6/Kip-Cip complexes. INK CKI binding releases the Kip/Cip CKIs associated to the cyclin D/CDK6/Kip-Cip complexes. In the G₀ and early G₁ phase, the retinoblastoma protein (pRb) is hypophosphorylated and sequesters the transcription factor complex E2F. Active cyclin D/CDKs and cyclin E/CDK2 phosphorylate pRb in mid-G₁ and late G₁ phase, respectively. Hyperphosphorylation of pRb disrupts its association to E2F. Once released, E2F activates the transcription of genes necessary to the G₁/S transition [7]. The hallmarks of quiescence are an increase in p27^{Kip1} and a decrease in the levels of cyclins, tightly inhibiting entry into the G₁ phase [8–10].

The three TGF- β isoforms belong to a superfamily of factors and exert a variety of effects, including stimulation/inhibition of cell growth, apoptosis or differentiation, extracellular matrix production and immunomodulation [11, 12]. On the cell membrane, TGF- β binds to the type II receptor, which recruits the type I receptor into the complex. The type I receptor is phosphorylated by the constitutive serine-threonine kinase activity of the type II receptor. When activated, the type I receptor phosphorylates Smad2 and Smad3, allowing them to heteromerize with Smad4 and translocate into the nucleus to regulate the transcription of target genes [11]. In addition, TGF- β has been shown to regulate other signaling pathways, e.g. the mitogen-activated protein kinases Erk, Jun N-terminal kinase, p38, phosphoinositide 3-kinase (PI3-K), PP2A phosphatases and Rho family members [13]. Unlike the canonical Smad pathway, these alternative pathways are activated by TGF- β in a cell-type-dependent manner and their biochemical links to the activated receptors are largely unknown.

TGF- β is predominantly described as an inhibitor of proliferation in epithelial and immune cells [11]. TGF- β inhibits cell cycle progression of epithelial cells by arrest in the G₁ phase [11]. It is well established that, in these cell types, p15^{INK4b} and p21^{Cip1} expression as well as an increase in p27^{Kip1} availability are induced by TGF- β and contribute largely to its ability to arrest cells in mid-G₁ or late G₁ phase by binding and inhibiting the complexes

cyclin/CDKs [14]. Moreover, TGF- β can inhibit CDK activity by down-regulating the CDK-activating kinases or the Cdc25C phosphatase [12]. However, in some mesenchymal cell lines, TGF- β can promote proliferation [15–18]. In cultured vSMCs, paradoxical results have been reported according to the confluence of the cells exposed to TGF- β . Most authors have agreed on the inhibition by TGF- β of mitogen-induced vSMC growth in subconfluent monolayers [19–30]. Interestingly, each time the growth of primary cultures of vSMC was compared in subconfluent versus confluent or post-confluent monolayers, it was shown that TGF- β promoted vSMC growth of confluent quiescent monolayers cultured either in mitogen-rich [25, 26, 29] or serum-deprived [22, 26, 31, 32] media. However, the mechanisms by which TGF- β exerts these opposing effects are not well understood.

The growth of vSMCs is also determined by the balance between cell proliferation and cell death by apoptosis [33–36]. Similar to proliferation, the impact of TGF- β on apoptosis is largely cell type and context dependent [37]. Data concerning the TGF- β effect on the apoptosis of cultured vSMCs are scarce and contradictory. vSMC apoptosis induced by serum deprivation was shown to be inhibited by TGF- β ₁ [35]. In another study [28], no TGF- β -induced apoptosis was observed in vSMCs cultured in mitogen-rich media. On the other hand, TGF- β induced apoptosis in vSMCs cultured in serum-free media [20, 38].

To investigate how TGF- β regulates cell proliferation and apoptosis according to cell density, the aim of the present study was to (i) reappraise in a rat vSMC line whether TGF- β actually shifts from a growth inhibitor to a growth inducer with increasing cell density, (ii) determine, in relation to cell density, the respective contribution of TGF- β -induced proliferation and apoptosis to the regulation of the cell number and (iii) analyze how TGF- β regulates cell cycle proteins and pro-apoptotic/pro-survival pathways to exert its density-dependent effects.

Materials and Methods

Cell Culture Protocols

A spontaneously transformed cell line of arterial smooth muscle cells, the V8 vSMC line, previously established in the laboratory from aortic media of adult Wistar rats [39, 40], was used in this study. The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, Lyon, France) containing 10% fetal bovine serum (FBS), L-glutamine, penicillin and streptomycin (Gibco BRL, Cergy-Pontoise, France) at 37°C in 95% air and 5% CO₂. Cell passages 15–21 were used. For the experiments, V8 vSMCs were plated in 6-well tissue culture plates at the indicated densities in DMEM with 10% FBS for 24 h to facilitate cell attach-

ment and growth. On day 0 (d_0), the cells were rinsed twice with phosphate-buffered saline (PBS) and were serum starved by switching to DMEM with 1% FBS. The following day (d_1), treatment started with DMEM with 1% FBS alone or supplemented with 1 ng/ml of TGF- β_1 (R & D Systems, Lille, France). The media were renewed daily. In preliminary experiments ($n = 3$), V8 vSMCs, plated at 0.05×10^6 cells/well, were treated or not with TGF- β_1 and the cells were counted from d_1 to d_6 . In experiments to investigate whether cell density modulated TGF- β_1 effects on V8 vSMCs ($n = 14$), control and TGF- β_1 -treated cells were seeded over a wide range of plating densities (0.05 – 0.6×10^6 cells/well) and studied at the end of d_3 . In each experiment, enough wells were prepared to be able to study several parameters (cell number, thymidine incorporation, Western blot and caspase-3 activity) on the same culture. In additional experiments to study the effects of TGF- β_1 on the phases of the cell cycle by flow cytometry ($n = 3$) and on the level of PI3-K activation ($n = 3$), control and treated cells having reached similar low or high densities on d_3 were compared.

Cell Counting

On the day of harvest, the cells were washed twice in PBS, dissociated with 500 μ l of trypsin/EDTA (Gibco BRL) and counted on a ZM Coulter counter (Coulter Electronics, Hialeah, Fla., USA). In each experiment, cell numbers were determined from triplicate wells for each plating density.

Thymidine Incorporation

The mitogenic activity of TGF- β_1 was assessed by determining the incorporation of [6 - 3 H]-thymidine (Perkin Elmer, Boston, Mass., USA) into DNA of control and treated cells at different final densities. The cells were pulse labeled during the last 4 h of d_3 with 1 μ Ci of [6 - 3 H]-thymidine in 1 ml of medium. The cells were washed twice with PBS, fixed with methanol/acetic acid (3:1, v/v) for 5 min at room temperature, washed once with the fixative and twice with 80% (v/v) methanol. Cells were then solubilized with 0.4% (w/v) sodium deoxycholate in 0.5 M NaOH. The incorporated radioactivity was measured in a scintillation counter. Triplicate wells were analyzed for each plating cell density.

Western Blot Analysis

At the end of d_3 , the cells were washed twice with PBS and lysed in 0.1 ml/well of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% (w/v) SDS, 100 mM NaF, 2 mM sodium orthovanadate and a protease inhibitor cocktail (Roche-Boehringer, Meylan, France). The protein concentration of each sample was determined by Lowry's method. Equal amounts of total proteins were separated under denaturing conditions by SDS-PAGE on 5–10% gradient gels (pRb), 10% gels (cyclins D $_1$ and E, caspase-3 and PI3-K p85) and 15% gels (p27 and p15) and transferred on PVDF membranes. The membranes were blocked in 5% non-fat dried milk in Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 0.1% Tween-20. The membranes were incubated overnight at 4°C using the following primary antibodies: anti-p27 (C-19, Santa Cruz Biotechnology, Le Perray-en-Yvelines, France), anti-p15 (K-18, Santa Cruz Biotechnology), anti-cyclin E (M-20, Santa Cruz Biotechnology), anti-cyclin D $_1$ (C-20, Santa Cruz Biotechnology), anti-Rb (14001A, BD Biosciences Pharmingen, Le Pont-de-Claix, France), anti-caspase-3 (611048, BD Biosciences Pharmingen), anti-phospho-(Tyr) p85 PI3-K (3821, Cell Signaling, Danvers, Mass., USA) and anti- β -actin (C-4, Santa Cruz Biotechnology).

After incubation with the respective horseradish-peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark), detection was achieved using an ECL chemiluminescence kit (Amersham-Pharmacia Biotech, Les Ulis, France) on Kodak X-omat AR films. Densitometric analysis of the blots was performed with the NIH Image software.

Caspase-3 Activity Assay

Apoptosis was quantified by measuring caspase-3 activity at the end of d_3 . The cells were rinsed twice with PBS and harvested with trypsin-EDTA. The cellular suspensions were collected and mixed with PBS containing 0.1% horse serum and centrifuged for 3 min at 3,000 rpm. The cell pellets were lysed with ice-cold hypotonic buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA and 0.01% Triton X-100). The lysates were subjected to two freeze-thaw cycles to optimize cell lysis and centrifuged at 3,000 rpm for 3 min to collect the supernatant. The activity of caspase-3 in the cytosolic extracts of each sample was assayed using the fluorogenic substrate Ac-DEVD-7-amino-4-methylcoumarin (AMC) at 50 μ M (Pharmingen, San Jose, Calif., USA). Ac-DEVD-AMC is proteolytically cleaved into free fluorescent AMC by caspase-3. After establishment of a standard curve with free fluorescent AMC in buffer reaction (20 mM HEPES, pH 7.5, 250 mM sucrose, 50 mM KCl, 2.5 mM MgCl $_2$ and 10 μ M DTT), caspase-3 activity was measured as emission events of the cleaved AMC fluorochrome (in pmol/min/mg protein). Fluorescence was measured by a Tecan Genious fluorimeter with a band-pass filter (excitation/emission: 465/360 nm).

Flow-Cytometric Analysis of Apoptosis and Cell Cycle

After 3 days in the presence or absence of TGF- β_1 , V8 vSMCs were pelleted and resuspended at a concentration of about 2×10^6 cells/ml for 10 min at room temperature in DNA staining solution I (50 μ g/ml propidium iodide, 100 U/ml RNase A, 0.1% Triton X-100 and 4 mM sodium citrate). An equal volume of DNA staining solution II (50 μ g/ml propidium iodide, 0.1% Triton X-100 and 400 mM NaCl) was added and the suspension was incubated in the dark at 4°C for at least 2 h prior to flow-cytometric analysis. Flow cytometry was performed on a Dako Galaxy flow cytometer. Laser excitation at 488 nm was produced by a 15-mW argon-ion laser. Fluorescence collection for propidium iodide was performed with 585/42 (FL2) band-pass filters. At least 10^4 events were analyzed per sample. The experiments were repeated at least 3 times. The doublet discrimination parameter was employed on integrated and peak DNA signals to exclude cell aggregates. The distribution of cells according to DNA content was used for the analysis of the cell cycle and for the determination of the percentage of subdiploid ($<2n$) cells. Cell cycle analysis was performed with the Flowmax software.

Statistical Analysis

Statistical analysis for more than two groups was performed using the StatView software for parametric tests (ANOVA) followed by Bonferroni's multiple comparison tests. In addition, ANOVA post hoc tests allowed pairwise testing for significant differences between TGF- β_1 -treated cultures versus controls. Otherwise, data analyses for two groups were performed using the unpaired Student's t test. Differences were considered significant for $p < 0.05$. Values were expressed as means $\pm$ SD or SEM.

Fig. 1. Kinetics of V8 vSMC growth in response to TGF-β₁. **a** V8 vSMCs were plated in 10 % FBS at 0.1×10^6 cells/well in triplicate and allowed to attach for 24 h. From d₀ (24 h after plating) to d₆, cells were cultured in DMEM with 1% FBS and then exposed or not to 1 ng/ml TGF-β₁ from d₁ to d₆. The medium and TGF-β₁ were renewed daily. Cells were counted electronically. Each point represents the mean $\pm$ SD of a representative experiment performed in triplicate. **b** V8 vSMCs were plated in DMEM with 10% FBS at the indicated plating density and allowed to attach for 24 h. On d₀ (24 h after plating), media were changed to DMEM with 1% FBS for 24 h, then the cells were exposed or not to TGF-β₁ from d₁ to d₃ in DMEM with 1% FBS. Cell numbers were determined at the end of d₃. Data are the means $\pm$ SEM from 14 experiments performed in triplicate. Significant differences between control cells and TGF-β₁-treated cells are indicated (* $p < 0.001$; ** $p < 0.0005$; *** $p < 0.0001$).

Results

TGF-β₁ Exerts a Density-Dependent Biphasic Effect on V8 vSMC Growth

Initially, V8 vSMCs, cultured in DMEM supplemented with 1% FBS in the absence or presence of TGF-β₁ (1 ng/ml), were seeded at 0.05×10^6 cells/well and cell counts were performed daily until d₆ (fig. 1a). In control cells, the growth curve of V8 vSMCs had a typical sigmoid shape and contact-inhibited cells reached the plateau phase on d₅. Under TGF-β₁ treatment, inhibition of cell growth was evident from d₂ and the cell number did not increase up to d₆. This protocol precluded the study of the effects of TGF-β₁ on the growth of confluent V8 vSMCs.

To determine the effect of TGF-β₁ on vSMC growth in relation to cell density, V8 vSMCs, seeded in 6-well culture plates at increasing densities (0.05×10^6 , 0.1×10^6 , 0.2×10^6 , 0.3×10^6 , 0.4×10^6 , 0.5×10^6 and 0.6×10^6 cells/well), were cultured in the absence or presence of TGF-β₁ (1 ng/ml) and cells were counted at the end of d₃ (fig. 1b). In control cells, the final cell number on d₃ augmented with increasing plating cell density up to 0.5×10^6 cells/well, after which they reached a plateau due to contact inhibition. In contrast, TGF-β₁-treated cells displayed a significantly slower growth rate than control cells seeded at densities $< 0.5 \times 10^6$ cells/well. Beyond a seeding density of 0.5×10^6 cells/well, TGF-β₁-treated cells continued to grow, reaching cell densities significantly higher than those observed in control cells at the plateau (fig. 1b). In TGF-β₁-treated cells seeded at high densities, no plateau was observed, indicating that dense cultures of V8 vSMCs treated with TGF-β₁ were no longer contact inhibited and could reach post-confluent densities.

TGF-β₁ Mediates a Density-Independent Steady Rate of DNA Synthesis in V8 vSMCs

Next, we investigated to which extent the biphasic effect of TGF-β₁ on V8 vSMC growth could be explained by the regulation of cell proliferation by determination of the incorporation of [6-³H]-thymidine (fig. 2a). The incorporation of [6-³H]-thymidine per cell during the last 4 h of d₃ was plotted as a function of the final cell densities on d₃ in control and TGF-β₁-treated V8 vSMCs (fig. 2a). Control cells displayed a rapid decrease in thymidine incorporation for increasing cell densities. At the highest cell densities tested, the levels of thymidine incorporation were practically negligible as a result of the quiescence induced by contact inhibition. In contrast, in the

Fig. 2. Effect of TGF- β_1 and cell density on the rate of V8 vSMCs proliferation. **a** At various cell densities, V8 vSMCs treated or not with TGF- β_1 for 3 days were subjected to thymidine incorporation studies. Cells were pulse labeled during the last 4 h of d₃ with 1 μ Ci/ml [³H]-thymidine. The numbers of cpm/well were divided by the numbers of cells. The cell numbers on d₃ from 14 independent experiments were grouped in classes centered on the values indicated on the x-axis. Values are means $\pm$ SEM. Significant differences between control cells and TGF- β_1 -treated cells are indicated (* $p < 0.005$; ** $p < 0.001$). In TGF- β_1 -treated cells, no significant difference was observed over the whole range of cell densities. **b** Phosphorylation of pRb in response to cell density and

TGF- β_1 . V8 vSMCs were cultured for 3 days in the presence or absence of 1 ng/ml TGF- β_1 . At the indicated cell density, control and TGF- β_1 -treated cells were lysed and proteins were analyzed by Western blot. Equal loading of proteins on the gels was confirmed by β -actin immunodetection. Protein samples were subjected to a 5–10% gradient SDS-PAGE. Western blot analysis was performed by probing the blots with a monoclonal antibody raised against pRb which recognizes both the hyperphosphorylated (ppRb) and hypophosphorylated (pRb) forms. Upper panels: representative Western blots. Lower panels: densitometric analysis from 5 independent experiments. Data are the means $\pm$ SEM of ppRb/pRb. Significant differences are indicated (* $p < 0.05$).

presence of TGF- β_1 , V8 vSMCs displayed low but steady levels of thymidine incorporation that were not significantly different over the full range of the cell densities tested. Comparison of the levels of thymidine incorporation in control and TGF- β_1 -treated cells at similar cell densities showed that TGF- β_1 significantly inhibited V8 vSMC proliferation at densities $< 0.6 \times 10^6$ cells/well. In contrast, TGF- β_1 stimulated the proliferation when V8 vSMCs reached densities $\geq 0.6 \times 10^6$ cells/well.

TGF- β_1 Imposes a Density-Independent Pattern of G₁- to S-Phase Progression to V8 vSMCs

We analyzed cell cycle distribution by flow cytometry in V8 vSMCs at low and high cell densities in the absence or presence of TGF- β_1 at the end of d₃ (table 1). In control vSMCs at low density, >15% of the cells were in the S phase of the cell cycle. As expected, in the absence of TGF- β_1 , high density induced a >5-fold significant decrease in the proportion of V8 vSMCs in the S phase of

the cell cycle and a significant increase in cells in G₁ phase. In contrast, under TGF- β_1 treatment, the proportion of V8 vSMCs in S phase was not significantly different between sparse and confluent cells. Comparison of control and TGF- β_1 -treated cells at equivalent low cell densities showed that TGF- β_1 reduced the fraction of cells in the S phase of the cell cycle >50% and concomitantly increased the fraction of cells in G₁ phase. In contrast, at high cell density, in TGF- β_1 -treated V8 vSMCs, the fraction of cells in S phase was twice that in control cells and the fraction of cells in G₁ phase was significantly lower in TGF- β_1 -treated cells than in control cells.

TGF- β_1 Enforces a Density-Independent, Constant Pattern of pRb Phosphorylation in V8 vSMCs

The molecular mechanisms by which TGF- β_1 exerted its density-independent regulation of proliferative activity in V8 vSMC were investigated. At first, we focused on the expression and phosphorylation levels of the retino-

Table 1. Cell cycle distribution of V8 vSMCs at low and high densities in response to TGF- β_1

		Cell cycle phase, %		
		G ₁	S	G ₂ /M
Low density	control	71.9 $\pm$ 2.9	15.5 $\pm$ 1.8	12.4 $\pm$ 1.9
	TGF- β_1	78.8 $\pm$ 1.3 ^a	7.1 $\pm$ 0.5 ^b	13.5 $\pm$ 1.1 ^c
High density	control	88.7 $\pm$ 0.7	2.9 $\pm$ 0.7	8.2 $\pm$ 1.2
	TGF- β_1	85.4 $\pm$ 0.9 ^d	6.4 $\pm$ 0.8 ^e	8 $\pm$ 1.1 ^f

Cells were plated at low and high cell densities in DMEM with 10% FBS for 24 h, switched to DMEM with 1% FBS for 24 h and then exposed or not to 1 ng/ml TGF- β_1 for 3 days, the media being changed daily. At the end of d₃, the cells were harvested and the DNA contents were analyzed by flow cytometry. The calculated percentages of cells in the G₀/G₁, S and G₂/M phases of the cell cycle are indicated. Values are the means $\pm$ SD of 3 separate experiments. Significant differences between control cells and TGF- β_1 -treated cells are indicated (^{a, b, d, e} $p < 0.05$; ^{c, f} $p > 0.05$; Student's unpaired t test).

blastoma protein (pRb), which is a common substrate for all cyclin/CDK complexes implicated in the early and late G₁ phase. Cyclin/CDK-induced pRb hyperphosphorylation converts pRb from a potent to an inactive suppressor of cell progression through G₁ and into S phase. Control V8 vSMCs at sparse densities that were actively proliferating displayed high amounts of hyperphosphorylated pRb (ppRb; fig. 2b, left upper panel). With the gradual increase in density in control cells, ppRb markedly and significantly decreased and hypophosphorylated Rb (pRb) remained unchanged. Consequently, the ppRb/pRb ratio diminished with increasing densities in control V8 vSMCs (fig. 2b, left lower panel). In sharp contrast, in TGF- β_1 -treated vSMCs (fig. 2b, left upper panel), ppRb and pRb were stable over the whole range of cell densities studied. As a result, the ppRb/pRb ratio did not change in response to TGF- β_1 when the cell density increased (fig. 2b, left lower panel). In control and TGF- β_1 -treated cells at equivalent densities, TGF- β_1 significantly decreased the ppRb/pRb ratio by 50% at low cell densities, while the opposite situation occurred at high cell densities (fig. 2b, right panels). It is noteworthy that the variations in pRb expression and the phosphorylation status with regard to cell density and to the presence or absence of TGF- β_1 were consistent with the results of thymidine incorporation and flow cytometry reported above.

TGF- β_1 Has Paradoxical Effects on G₁- and G₁/S-Regulatory Proteins in V8 vSMCs

Then, we investigated the expression levels of regulatory proteins that play crucial roles in the progression of the G₁ phase of the cell cycle.

At first, using Western blot, we studied two of the main regulators of the early G₁ phase, the CKI p15^{INK4b} and cyclin D₁. Control and TGF- β_1 -treated cells contained similar p15^{INK4b} levels that increased with cell density and similar cyclin D₁ levels that did not change with cell density (data not shown).

We next analyzed the protein levels of two important regulators of the late G₁ phase, p27^{Kip1} and cyclin E. The CKI p27^{Kip1} binds to and inhibits the activity of cyclin E-CDK2 complexes. In control cells, p27^{Kip1} protein levels dramatically increased with confluence. Surprisingly, in the presence of TGF- β_1 , the p27^{Kip1} protein levels remained low and constant over the whole range of cell densities tested (fig. 3a, left panels). At low cell density, the amounts of p27^{Kip1} were not significantly different between TGF- β_1 -treated cells and control cells, whereas at high cell density the amounts of p27^{Kip1} were markedly and significantly lower in TGF- β_1 -treated cells than in control cells (fig. 3a, right panels).

Finally, we studied the expression of cyclin E, an essential regulator of CDK2 activity that controls cell cycle progression through the G₁/S transition. In control cells, the amounts of cyclin E displayed a steep and significant decrease at cell densities as low as 0.2×10^6 cells/well and reached a nadir at confluence (fig. 3b, left panels). In contrast, in TGF- β_1 -treated cells, the decline in cyclin E protein was delayed until the cell density reached $0.5\text{--}0.7 \times 10^6$ cells/well (fig. 3b, left panels). Consequently, at low as well as high cell densities (fig. 3b, right panels), TGF- β_1 -treated cells contained much greater amounts of cyclin E than control cells.

TGF- β_1 -Induced Apoptosis in V8 vSMCs Is Inhibited by Increasing Cell Density

Observation of TGF- β_1 -treated cells at low densities (data not shown) suggested that detached cells might undergo apoptosis. This was confirmed in flow-cytometric experiments by measuring the proportion of cells with subdiploid DNA contents (fig. 4a). At low densities, the percentage of subdiploid cells, being very low in control cells, was significantly increased in TGF- β_1 -treated cells (12 ± 1 vs. $1.7 \pm 0.2\%$, $p < 0.001$). At high densities, TGF- β_1 lost its pro-apoptotic effect, which is reflected by the very small percentages of subdiploid cells in TGF- β_1 -treated and control cells (2 ± 0.2 vs. $1.9 \pm 0.36\%$, $p > 0.05$).

Fig. 3. Effect of TGF- β_1 on the regulatory proteins of the G₁ phase as a function of V8 vSMC density. V8 vSMCs were cultured for 3 days in the presence or absence of 1 ng/ml TGF- β_1 . At the indicated cell density, control and TGF- β_1 -treated cells were lysed and proteins were analyzed by Western blot. **a** The effect of TGF- β_1 on p27^{Kip1} expression as a function of V8 vSMC density. The samples were resolved on 10% acrylamide gels. The blots were probed with a polyclonal antibody specific for p27^{Kip1}. Upper panels: representative Western blots. Lower panels: in the densitometric analysis the data represent the means $\pm$ SEM from 4 independent experiments. In the lower left panels, the data are presented as

percent changes from the values at the lowest cell density after normalization to β -actin. Significant differences are indicated (* $p < 0.05$). **b** The effect of TGF- β_1 on cyclin E expression as a function of V8 vSMC density. The samples were resolved on 10% acrylamide gel. The blots were probed with a polyclonal antibody specific for cyclin E. Upper panels: representative Western blots. Lower panels: in the densitometric analysis, the data represent the means $\pm$ SEM from 4 independent experiments. In the lower left panels, the data are presented as percent changes from the values at the lowest cell density after normalization to β -actin. Significant differences are indicated (* $p < 0.05$).

Fig. 4. Effect of TGF- β_1 on apoptosis and PI3-K activity as a function of V8 vSMC density. V8 vSMCs treated or not with TGF- β_1 for 3 days were studied at the end of d₃ at various densities. **a** Effect of TGF- β_1 on apoptosis as a function of V8 vSMC density. At the end of d₃, the cells were stained with propidium iodide and analyzed by flow cytometry. The distribution of the cells according to their DNA content is shown. The percentage of subdiploid (apoptotic) cells was calculated and is indicated. Results are the means $\pm$ SD of at least 3 independent experiments. **b** Effect of TGF- β_1 on caspase-3 activity as a function of V8 vSMC density. At the end of d₃, caspase-3 activity was assayed on the cell lysates. The values of the assays were divided by the cell number on d₃. Values are the means $\pm$ SEM. Significant differences between control and TGF- β_1 -treated cells are indicated (* p < 0.005; ** p < 0.001). **c** Effect of TGF- β_1 on caspase-3 activation as a function of V8 vSMC density. The samples were resolved on 10% acryl-

amide gels. The blots were probed with a monoclonal antibody recognizing both procaspase-3 and the active cleaved caspase-3. Equal loading of proteins was confirmed by β -actin immunodetection. Upper panels: representative blots. Lower panels: densitometric analysis from 4 independent experiments. The data are expressed as means $\pm$ SEM of the ratio of cleaved caspase-3 to procaspase-3. **d** Effect of TGF- β_1 on PI3-K activation as a function of V8 vSMC density. The samples were resolved on 10% acryl-amide gel. The blots were probed with a polyclonal antibody recognizing the active regulatory subunit of PI3-K (phospho-p85). Equal loading of proteins was confirmed by β -actin immunodetection. Upper panels: representative blots of phospho-p85. Lower panels: densitometric analysis of 4 independent experiments. The data are means $\pm$ SEM after normalization to β -actin. Significant differences are indicated (* p < 0.05).

Caspases are key components of cell death pathways and, among them, caspase-3 is crucial, because it is activated by numerous death signals including TGF- β_1 . As described in figure 4b, in untreated V8 vSMCs caspase-3 activity was negligible and remained unchanged over the whole range of cell densities tested, whereas in TGF- β_1 -treated cells caspase-3 activity was prominent at low cell density and decreased steadily while cell density increased to become negligible at high density.

By Western blotting (fig. 4c), in comparison to control cells, TGF- β_1 significantly induced cleavage and thus activation of procaspase-3 at low densities. In contrast, at high cell density, TGF- β_1 lost its ability to cleave procaspase-3, which was in agreement with the inhibition of caspase-3 activity reported above.

TGF- β_1 Inhibition of the PI3-K Survival Pathway Is Overridden by Increasing Cell Density

To explain the low serum requirement for V8 vSMC growth, their refractoriness to apoptosis induced by serum deprivation and the modulation of apoptotic activity by TGF- β_1 , we investigated an important survival pathway, the PI3-K/Akt pathway. The levels of activation of the p85 subunit of PI3-K (phospho-p85) were studied by Western blot. It is noteworthy that the phosphorylation level of p85 increased markedly with increasing cell density in control cells (fig. 4d, left panels). Compared to their corresponding controls, TGF- β_1 -treated V8 vSMCs showed an inhibition of the phosphorylation level of p85 at low densities (fig. 4d, middle panels), whereas the inverse situation prevailed at high densities, i.e. the level of phospho-p85 being markedly stimulated (fig. 4d, right panels).

Discussion

In this study, we used a spontaneously transformed cell line of arterial smooth muscle cells established from aortic media of adult Wistar rats, the V8 vSMCs [39, 40]. These cells have an epithelioid or cobblestone appearance, they retain the expression of α -smooth muscle actin, they are highly proliferative even under conditions of serum deprivation, and they do not display the classical hill-and-valley pattern of growth observed at confluence in primary cultures of vSMCs but still form a monolayer at high density. Such features, typical of the synthetic phenotype of vSMCs in vitro, have been described in other vSMC lines [41, 42]. Our study shows that the V8 vSMC line displays growth kinetics in response to TGF-

β_1 and cell density being quite similar to that described in primary cultures of murine aortic vSMCs [22, 25, 26, 29, 31, 32]. Despite their differences with primary cultures of vSMCs, the V8 vSMC line is a relevant model to study the bimodal effects of TGF- β on vSMC growth and explore the mechanisms involved. One mechanism proposed to explain the stimulation of the growth of confluent primary vSMCs was TGF- β -induced autocrine production of PDGF-AA [26]. However, reexamination of this hypothesis showed that the production of PDGF-AA could only account for 10–40% of the proliferation of confluent vSMCs induced by TGF- β [32]. Alternatively, TGF- β was suggested to exert opposite effects on vSMC proliferation via interaction with distinct receptor subtypes selectively expressed as a function of cell density [22]. This last hypothesis was not experimentally assessed. Very recently, three investigations on the mechanisms of the growth inhibition of subconfluent vSMC induced by TGF- β were conducted. First, studies in primary cultures of aortic vSMCs from Smad3 $^{-/-}$ mice led to the conclusion that vSMC growth inhibition by TGF- β was, at least in part, dependent on Smad3 [24]. In another study on primary cultures of aortic vSMCs from wild-type rats [28], TGF- β -induced growth inhibition was essentially p38 dependent. In the last study [43], TGF- β could inhibit vSMC proliferation by repressing the transcription of the cyclin A gene, another cell cycle protein that facilitates the G₁- to S-phase transition by complexing with and activating CDK2. In these last three reports, the effects of TGF- β on confluent vSMCs were not studied. In summary, to date, no satisfactory mechanisms have been proposed to explain the bimodal effect of TGF- β on vSMC growth as a function of cell density. In the present report, unconventional regulations of G₁-phase-regulatory proteins and apoptosis provide coherent explanations for the density-dependent opposite effects of TGF- β on V8 vSMC growth.

In control V8 vSMCs, as in most other cell types, increasing cell density triggered quiescence by contact inhibition and the growth curve reached a plateau. Apoptosis was not involved in the regulation of growth of untreated V8 vSMCs. With increasing cell density, untreated V8 vSMCs displayed a dramatic decrease in the level of thymidine incorporation, the percentage of cells in the S phase of the cell cycle and the level of hyperphosphorylated Rb. This was confirmed at the molecular level since untreated V8 vSMCs recapitulated the classical steps of growth inhibition and contact inhibition induced by confluence. In sparse untreated V8 vSMCs, cyclin D₁ and in particular cyclin E levels were high while amounts

of p15^{INK4b} and p27^{Kip1} were low supporting the activation of the cyclin D/CDKs and cyclin E/CDK2 complexes, which was coherent with the very high level of hyperphosphorylated pRb. In confluent untreated vSMCs, levels of cyclin D₁ were unchanged and those of cyclin E sharply decreased, while p15^{INK4b} and notably p27^{Kip1} levels increased. As a result, cyclin D/CDKs and above all cyclin E/CDK2 should be tightly inhibited, which led to the extremely low levels of hyperphosphorylated pRb observed at high densities. Both the very high increase in p27^{Kip1} expression and the very sharp decrease in cyclin E at confluence suggest that p27^{Kip1} and the cyclin E/CDK2 complexes have a central role in the inhibition of the mitotic activity in response to increasing cell density and in induction of quiescence by contact inhibition in V8 vSMCs.

TGF- β_1 dramatically altered the growth curve of V8 vSMCs. Over the whole range of seeding densities, the cell numbers constantly increased and the curve did not reach a plateau. Our results show how the control exerted by TGF- β_1 on V8 vSMC proliferation, contact inhibition and apoptosis induced these alterations in the shape of the growth curve. The reshaping of the V8 vSMC growth curve by TGF- β_1 was mainly exerted through its control of cell proliferation. Unexpectedly, TGF- β_1 imposed a low but constant level of proliferation on V8 vSMCs at all cell densities tested. This was confirmed by three independent methods: thymidine incorporation, cell cycle analysis and phosphorylation status of pRb. Compared with untreated cells, which display a striking decrease in proliferation when cell density increased, the low but constant level of mitotic activity induced by TGF- β_1 is sufficient to explain how TGF- β_1 inhibits proliferation at low densities and stimulates proliferation at high densities. Remarkably, the TGF- β_1 -induced low level of proliferation was maintained in cells cultured at high densities allowing them to reach post-confluent densities. This implies that TGF- β_1 was able to relieve V8 vSMCs from contact inhibition that is responsible for quiescence in untreated confluent cells. Control of apoptosis by TGF- β_1 also took part in the reshaping of the V8 vSMC growth curve. At low cell densities, active apoptosis and low proliferative activity concurred to the decrease in cell numbers observed in TGF- β_1 -treated cells compared to controls. In contrast, at high densities, with TGF- β_1 having lost its pro-apoptotic effect, TGF- β_1 -induced stimulation of proliferation was unopposed compared with control cells. The molecular mechanisms used by TGF- β_1 to induce a constant level of proliferation in V8 vSMCs were very unconventional. Up-regulation of p15^{INK4b}, consid-

ered as central in the TGF- β -induced cytostatic program [12], did not occur in V8 vSMCs since p15^{INK4b} levels remained comparable to those in control cells at all densities. Whereas an increase in p27^{Kip1} bioavailability is the expected cell response to TGF- β_1 [12], TGF- β_1 maintained in V8 vSMCs a constant density-independent low level of p27^{Kip1} protein so that TGF- β_1 opposed the dramatic increase in p27^{Kip1} protein that occurred in control cells rendered quiescent by confluence. In addition, TGF- β_1 exerted unexpected regulations on the cyclin levels in V8 vSMCs. At all cell densities, cyclin D levels were similar in cells treated or not with TGF- β_1 while TGF- β_1 induced cyclin E levels that were higher than in control cells. At sparse densities, a low p27^{Kip1} level and a relative cyclin E excess are disconnected from the proliferative activity of TGF- β_1 -treated cells that is lower than that of control cells. Additional studies will be required to investigate whether inhibition of the levels and/or activation of the CDKs are involved in the tuning of the rate of proliferation in V8 vSMCs by TGF- β_1 . On the other hand, our study provides sound clues pertaining to the mechanisms by which TGF- β_1 allowed V8 vSMCs to escape contact inhibition and proliferate at high cell densities. p27^{Kip1} overexpression is a hallmark of cellular quiescence induced by serum starvation or contact inhibition [8]. Our demonstration of the inhibition of the dramatic accumulation of p27^{Kip1} protein by TGF- β_1 occurring in control cells with confluence provides evidence of the escape of confluent vSMCs from contact inhibition. Low p27^{Kip1} levels associated with high cyclin E levels compared with control cells allow confluent TGF- β_1 -treated vSMCs to proliferate and reach post-confluent densities. Similar to V8 vSMCs, the proliferation of confluent C3H 10T1/2 fibroblasts was stimulated in response to TGF- β_1 treatment [17]. Interestingly, in this cell line, the underlying mechanism of proliferation increase compared with control cells was shown to be due to the down-regulation of p27^{Kip1} and the activation of the cyclin E/CDK2 complexes. Recent evidence supports our finding that TGF- β_1 down-regulates p27^{Kip1} in a vSMC line [43]. In this study, a decrease in p27^{Kip1} protein was observed in sparse A10 vSMCs treated for 24 h by TGF- β_1 . Whether TGF- β_1 had the same effect on confluent cells was not investigated in this study.

Another original finding of our study was the contribution of apoptosis to the regulation of V8 vSMC growth in response to TGF- β_1 and cell density. The V8 vSMCs were refractory to apoptosis induced by serum deprivation. This is a characteristic of the V8 vSMC line that has not been reported yet. Since the high activity of PI3-K

and thus of protein kinase B (Akt) protects very efficiently against apoptosis [44, 45], a plausible explanation for the refractoriness of V8 vSMCs to apoptosis is their high basal level of PI3-K activation shown in this study. Such a high PI3-K/Akt activity might also explain the low serum requirement for growth of the V8 vSMCs. The proapoptotic effect of TGF- β_1 was restricted to V8 vSMCs cultured at low densities. The increase in caspase-3 activity in TGF- β_1 -treated cells observed at low density was correlated with the activation of procaspase-3 into its cleaved and active form. Such an activation of caspase-3 by TGF- β_1 has been reported previously in different cellular models [46–48]. It is noteworthy that TGF- β_1 -induced apoptosis in sparse V8 vSMCs is linked to a decrease in PI3-K activation that inhibits the pro-survival PI3-K/Akt pathway. Our finding is in line with two reports showing that TGF- β can induce apoptosis through inhibition of Akt, a direct downstream target of PI3-K. In hematopoietic cells [49] and mammary epithelial cells [46], TGF- β induces apoptosis by inhibiting Akt phosphorylation and activity. In contrast, the level of PI3-K activation in V8 vSMCs is strongly increased by TGF- β_1 at high cell densities and, concomitantly, TGF- β_1 loses its apoptotic effect. This is in agreement with several reports showing that up-regulation of PI3-K or its direct downstream target Akt protects against TGF- β -induced apoptosis [50–53]. It has been shown that physical interactions between activated Akt and Smad3 inhibit the Smad3-dependent apoptotic cascade induced by TGF- β [51, 53]. A puzzling question is how high cell density not only suppressed the TGF- β_1 -induced inhibition of PI3-K observed at low cell density but also resulted in higher levels of PI3-K activation compared with those observed in confluent untreated cells. A combination of the effects of TGF- β_1 and cell density provides potential mechanisms that could explain the stimulation of the pro-survival PI3-K/Akt pathway at high density. It is known that cell-cell contacts activate two intercellular adhesion molecules, β -catenin/N-cadherin and Notch that mediate signaling via the PI3-K/Akt pathway in vSMCs [54, 55]. Interestingly, the enhanced PI3-K activity observed in the V8 vSMCs when cell density increased is in line with these studies. It is reasonable to postulate that the post-confluent densities reached by TGF- β_1 -treated V8 vSMCs increased cell-cell interactions and thus PI3-K/Akt activity to a greater extent than in mere confluent control cells. In addition, PI3-K/Akt activation could be further stimulated by TGF- β_1 if TGF- β_1 is able to up-regulate the expression of N-cadherin and intracellular mediators of the Notch signaling pathway in V8 vSMCs, as it does in oth-

er cell types [56, 57]. In line with these data, the resistance to apoptosis of vSMCs overexpressing a direct downstream target of Notch, HRT1, was associated to the induction of the expression and activity of the PI3-K/Akt pathway [55]. Thus, the activity of the PI3-K/Akt pathway is likely to be increased by these two intercellular signaling pathways positively regulated by TGF- β_1 . Our data fit well with the involvement of cell-cell interactions in V8 vSMC suppression of TGF- β_1 -induced apoptosis. Alternatively, at high cell density, TGF- β_1 could promote the PI3-K/Akt pathway by increasing cell-matrix anchorage [58] or stimulation of the synthesis of growth factors [59].

To conclude, TGF- β_1 and cell density regulate the growth of V8 vSMCs through divergent modulations of cell proliferation and apoptosis. Our observations show that TGF- β_1 dictates a low but constant rate of proliferation even at high density, which allows the cells to escape contact inhibition, which occurs via an unconventional regulation of the p15^{INK4b}, p27^{Kip1} and cyclin E levels. In contrast, active apoptosis induced by TGF- β_1 at low cell densities is antagonized at high cell density, and the level of apoptosis is inversely correlated to PI3-K activation. To confirm the relevance of these data in the control of vSMC growth in the intima and media in vivo will be the focus of our future work in transgenic mice in which the TGF- β signaling pathway will be invalidated in vSMCs.

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