Research Paper

Journal of Vascular Research

J Vasc Res 2006;43:157–165
DOI: 10.1159/000090945

Received: August 19, 2005
Accepted after revision: October 16, 2005
Published online: January 11, 2006

Serum Starvation and Growth Factor Receptor Expression in Vascular Smooth Muscle Cells

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Key Words
Atherogenesis · IGF-1 receptor · PDGFβ receptor · Serum starvation · Smooth muscle cells

Abstract
Background: Smooth muscle cell (SMC) proliferation in atherosclerosis is regulated through the interaction of growth factors like platelet-derived growth factor-BB (PDGF-BB) and insulin-like growth factor-1 (IGF-1) and their receptors (R). We hypothesized that serum starvation of SMCs may affect PDGFβ-R and IGF-1-R expression and, consequently, the effect of their cognate ligands on SMC survival/proliferation. ***Methods and Results:*** Serum starvation significantly increases PDGFβ-R but not IGF-1-R mRNA and protein expression in SMCs. PDGF-BB stimulates cell survival but not proliferation in serum-starved SMCs of the synthetic phenotype, whereas SMCs of the contractile phenotype respond to PDGF-BB by a significant increase in proliferation. Immunohistochemical analysis of coronary atherosclerotic lesions reveals PDGFβ-R expression in SMCs in the lamina fibromuscularis, but not in the media and in healthy parts of the arterial wall. No such differential expression was observed for IGF-1-R. ***Conclusions:*** Differential regulation of PDGFβ-R and IGF-1-R expression by serum starvation might represent a mechanism for the control of SMC survival/proliferation in atherogenesis and restenosis. The distribution of PDGFβ-Rs and IGF-1-Rs in atherosclerotic lesions may indicate an effect of serum starvation on SMCs in the arterial wall.

Introduction

Smooth muscle cell (SMC) proliferation is a key event in the formation of atherosclerotic lesions and the development of restenosis following angioplasty [1, 2]. The lamina fibromuscularis of human medium-sized arteries is obviously a crucial place for the induction of SMC proliferation in either atherogenesis or restenosis [1–4].

SMC proliferation is tightly regulated through the interaction of growth factor receptors on the SMC surface and their cognate ligands [5–7]. Among these growth factors, platelet-derived growth factor-BB (PDGF-BB) and insulin-like growth factor-1 (IGF-1) are centrally involved in the control of SMC proliferation [8–15]. PDGF-BB

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and IGF-1 act on cell cycle in a different manner. Simultaneous addition of PDGF-BB and IGF-1 to SMCs induces synergistic effects on DNA incorporation and mitosis in vitro [16, 17]. Thus, the combined effects of PDGF-BB and IGF-1 on vascular SMCs are considered to be physiologically more relevant than their individual actions [18].

Both, PDGF-BB and IGF-1 are present in atherosclerotic lesions [5–10]. Their mitogenic activity is mediated by high-affinity binding to their corresponding receptors designated PDGFβ receptor (PDGFβ-R) and IGF-1 receptor (IGF-1-R) [14, 15]. Differential expression of these growth factor receptors in human atheroma might be essential for the proliferative response of SMCs in atherogenesis and restenosis. Studies in animal models suggest that balloon injury influences PDGFβ-R expression depending on the time course after injury [19] and that PDGFβ-R overexpression in aortas of diabetic animals might be crucial for the development of intimal hyperplasia [20]. However, data on their regulation in human arteries are lacking.

Diffusion tracer studies of atherosclerotic arteries revealed that the arterial lamina fibromuscularis is a place of starvation [21, 22]. Since the elastic lamina between intima and media represents a diffusion barrier and since the lamina fibromuscularis itself is not vascularized, nutritional supply of the inner part of the arterial intima is only guaranteed by diffusion from the arterial lumen and is directly dependent on its thickness [21, 22]. Serum starvation, through an increase in arterial wall thickness, might therefore locally influence cell function in the course of atherogenesis. Interestingly, in human medium-sized arteries, this deeper part of the intima next to the media represents the location of scattered SMCs [23], suggesting low nutritional supply of these cells in arteries with a thickened, atherosclerotic intima.

We hypothesized that serum starvation [24, 25] might regulate SMC growth factor receptor expression in vitro. This may reflect in vivo conditions where thickness and thus nutritional support in human arteries might influence the expression of growth factor receptors on human SMCs.

Methods

Cell Culture

SMCs were isolated from tissue explants of human internal mammarian arteries by the explant method [26, 27]. The secondary cultures of human SMCs were maintained in DMEM containing 20% FCS, fed once every other day, and split to a ratio of 1:2 upon reaching confluence. Cells were dissociated using a trypsin/EDTA solution (GIBCO, Karlsruhe, Germany) in PBS. Subcultures 5–9 were used and cultured in DMEM containing 20% FCS and antibiotics. Human coronary artery (CA) SMCs and recommended culture medium, consisting of smooth muscle basal medium (BM/5% FCS) were purchased from Cambrex, Walkersville, USA. Cells were demonstrated to be SMCs rather than fibroblasts by staining with an antibody to SMC α-actin and by assessing cell morphology and growth characteristics.

CA Specimens

CA specimens were prepared from human hearts obtained at autopsies. Five specimens showing advanced atherosclerotic lesions were selected for analysis as characterized by disorganization of the structure of the intima and changes in the inner contour of the arterial segment [23]. Serial transverse sections of 4–5 μm thickness were used for immunohistochemistry as described [28, 29].

Antibodies for Immunohistochemical Staining

A murine monoclonal antibody (Calbiochem, Darmstadt, Germany) recognizing the extracellular portion of PDGFβ-R and not cross-reacting with PDGFα-R[30] was used at a concentration of 20 μg/ml. An antibody against smooth muscle α-actin was purchased from PROGEN, Heidelberg, Germany (clone ASM-1) and used at a concentration of 0.5 μg/ml. The antibody against IGF-1-R (chicken polyclonal IgG) specific to the IGF-1-R β-subunit [31] (Upstate Biotechnology, Hamburg, Germany) was used at a concentration of 25 μg/ml. Primary antibodies were detected using biotinylated polyclonal antibodies (Vector Laboratories, Burlingame, USA). Isotype-matched, irrelevant antibodies were selected as controls.

Immunohistochemical Staining

Slides were treated with primary antibody for 1 h and then incubated with biotin-conjugated antibody and, consecutively, with avidin-biotin-peroxidase. Reaction products were revealed by immersing slides in 3-amino-9-ethylcarbazole to give a red-colored reaction product. Finally, slides were counterstained with hemalaun and mounted [29]. In negative controls, the primary antibody was replaced by PBS or irrelevant immunoglobulins.

RNA Extraction and Northern Blot Analysis

For mRNA analysis of PDGFβ-R and IGF-1-R expression, total RNA from SMCs was extracted using the RNeasy RNA extraction kit (Qiagen, Hilden, Germany) and used for Northern blot analysis as described before [32]. cDNA probes were generated by RT-PCR using the following primer sequences: IGF-1-R (sense 5′-CTG AAG CCC TGG ACT CAG TAC G-3′ and antisense 5′-GGA AAT TCT CAA AGA CTT TGC GG-3′), and PDGFβ-R (sense 5′-TGA CCA CCC AGC CAT CCT TC-3′ and antisense 5′-GAG GAG GTG TTG ACT TCA TTC-3′).

Western Blot Analysis

Western blot analysis for PDGFβ-R and IGF-1-R expression was performed on whole lysates of SMCs as described [32]. Membranes were incubated with goat antibodies to IGF-1-Rβ and PDGFβ-R (Santa Cruz, San Diego, Calif., USA) for 1 h. Osteopontin was detected by a goat polyclonal antibody (Santa Cruz) raised against a peptide mapping near the carboxy terminus of osteopontin of human origin. Osteopontin (Sigma, St. Louis, Mo., USA) was

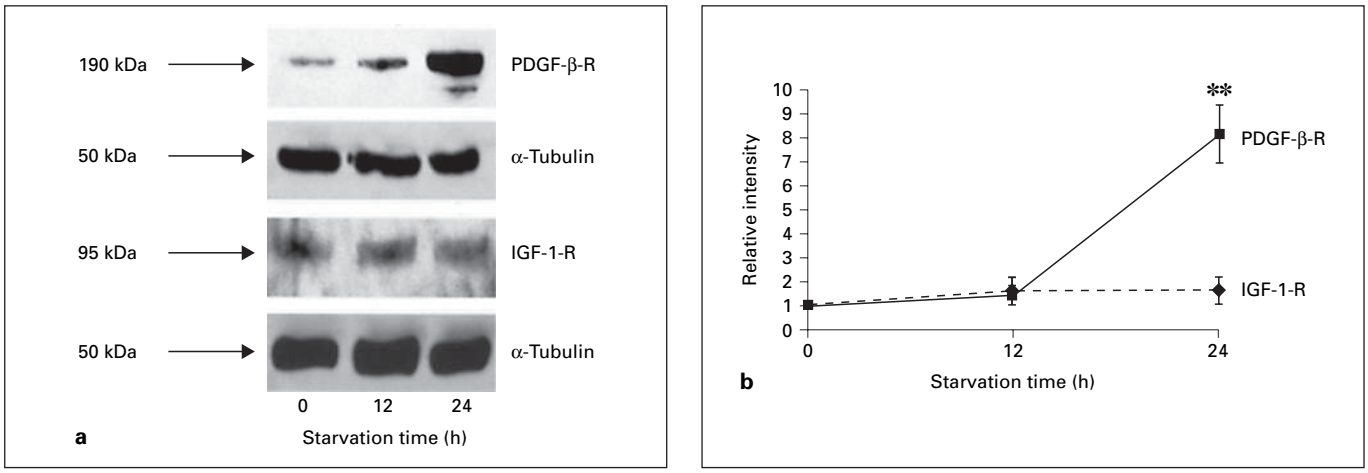

Fig. 1. Effect of serum starvation on PDGFβ-R and IGF-1-R protein expression in SMCs. Cells were grown in DMEM/20% FCS to 70% confluence, serum starved, and Western blot analysis on PDGFβ-R and IGF-1-R was performed at the times indicated. Equal loading of intact protein in all samples was verified by α-tubulin expression. **a** Representative experiment demonstrating a marked increase in SMC PDGFβ-R expression over time of serum starvation and the lack of an effect on IGF-1-R-expression. **b** Densitometric analysis of six independent experiments demonstrating a significant 8.6 ± 2.1-fold increase in PDGFβ-R protein expression (** $p < 0.01$ vs. unstarved cells, unpaired test). Serum starvation had no significant effect on IGF-1-R expression.

used as a positive control. Membranes were then incubated with horseradish-peroxidase-conjugated rabbit anti-goat antibody. Antigen detection was performed with a chemiluminescent detection system (Amersham, Braunschweig, Germany). Protein expression was quantified by densitometric analysis. Equal loading of intact protein was verified by α-tubulin expression.

[^{3}H]-Thymidine Incorporation

SMCs were plated in 24-well plates. After reaching a cell confluence of 70–80%, culture medium was changed to 0.2% FCS for 24 h. Cells were then stimulated with 20 ng/ml PDGF-BB in DMEM or BM, respectively, for 24 h. During the final 4 h, each well was further incubated with 5 μl [^{3}H]-thymidine at 37°C. The supernatant was then removed and 1 ml of a 5% trichloroacetic acid solution was added. SMCs were placed on ice for 30 min. After washing with double-distilled H_2O, 400 μl of 1 *M* NaOH was added for 10 min. Finally, 400 μl of 2 *M* HCl and 10 ml scintillation solution (Ultima Gold, PerkinElmer Life and Analytical Sciences, Boston, Mass., USA) were added. One hour later, radiation was measured using a β-counter (Wallac 1410; Pharmacia, Helsinki, Finland).

Cell Count

SMC proliferation/survival was assessed by counting total cell numbers. SMCs were plated in 24-well plates. After reaching a cell confluence of 70–80%, the incubation medium was changed to 0.2% FCS for 24 h. Cells were then stimulated with 20 ng/ml PDGF-BB in DMEM or BM, respectively, for 48 h. Cell count was performed according to the manufacturer's instructions using an electronic cell counter (CASY®1; Schärfe Systems, Reutlingen, Germany).

Statistical Analysis

Statistical analysis was performed using SigmaStat (version 2.0) software. Results were expressed as means ± SEM. Tests included the t test, one-way analysis of variance and the Mann-Whitney rank sum test. Tests were calculated two-sided. Paired tests were performed for follow-up within groups, and unpaired tests for comparisons between groups. Statistical significance was assumed in case of a p value <0.05; $p < 0.001$ was considered as highly significant.

Results

Serum Starvation Upregulates PDGFβ-R but Not IGF-1-R Protein Expression in Human SMCs in vitro

To investigate the effect of serum starvation on growth factor receptor expression in SMCs we used an established model of serum depletion [24, 25] and performed Western blot analysis on PDGFβ-R and IGF-1-R. SMCs were seeded in 75-cm^2 flasks and maintained in DMEM/20% FCS. After reaching 70% confluence, medium was changed to starvation medium (DMEM/0.2% FCS), and cells were cultured in this medium at the times indicated. As shown in figure 1a, serum starvation upregulates PDGFβ-R protein expression in a time-dependent manner, with a maximum after 24 h. Densitometric analysis of six independent experiments revealed a 8.6- ± 2.1-fold increase in PDGFβ-R protein expression

($p < 0.01$ vs. unstarved cells; n = 6, fig. 1b). In contrast, serum starvation had no effect on IGF-1-R expression in SMCs (fig. 1), suggesting a differential effect of serum starvation on growth factor receptor expression in SMCs. Equal loading of intact protein in all samples was verified by α-tubulin expression.

Serum Starvation Upregulates PDGFβ but Not IGF-1-R mRNA Expression in Human SMCs in vitro

To assess whether the effect of serum starvation on growth factor receptor protein expression was due to changes in PDGFβ-R and IGF-1-R mRNA expression, SMCs were treated as described above, and Northern blot analysis was performed at different time points. Corroborating the data obtained by Western blot analysis, serum starvation upregulated PDGFβ-R mRNA expression in a time-dependent manner but had no effect on mRNA expression of IGF-1-R (fig. 2). Equal loading of intact RNA was confirmed by investigating glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression.

PDGF-BB Downregulates Starvation-Induced Increase in PDGFβ-R Expression

To assess the role of the above-employed growth factors in the expression of their corresponding receptors, cells were starved for 24 h and stimulated with PDGF-BB (10 ng/ml) or IGF-1 (10 ng/ml), respectively. Northern blot analysis was performed at the times indicated. As depicted in figure 3a, addition of PDGF-BB to serum-

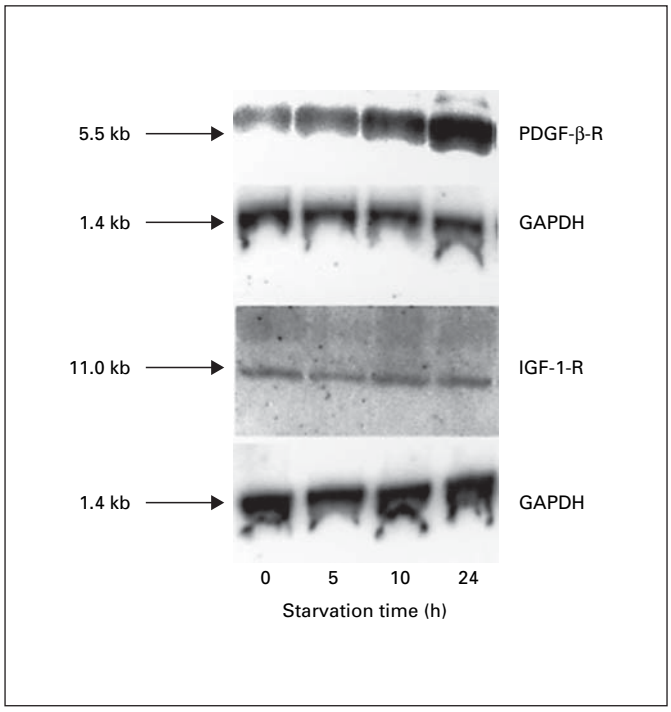

Fig. 2. Effect of serum starvation on PDGFβ-R and IGF-1-R mRNA expression in SMCs. Cells were grown in DMEM/20% FCS to 70% confluence, serum starved, and Northern blot analysis on PDGFβ-R and IGF-1-R mRNA was performed at the times indicated. Equal loading of intact RNA was confirmed by GAPDH mRNA expression. Representative experiment demonstrating a marked increase in SMC PDGFβ-R mRNA expression over time of serum starvation and the lack of an effect on IGF-1-R mRNA-expression. Three independent experiments yielded similar results.

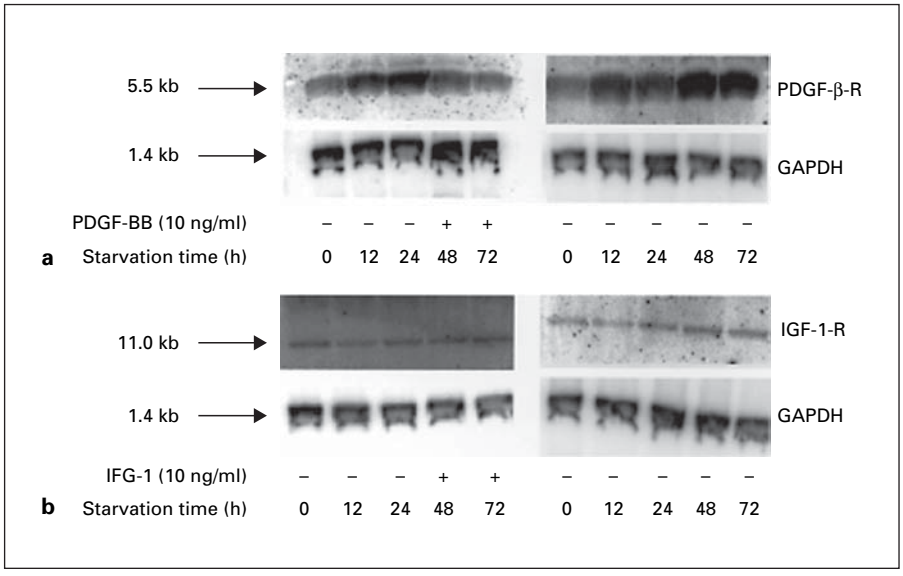

Fig. 3. Effect of PDGF-BB and IGF-1 on mRNA expression of their corresponding receptors. After 24 h of serum starvation PDGF-BB (10 ng/ml) or IGF-1 (10 ng/ml) were added to SMCs, respectively. **a** Effect of PDGF-BB addition (left panel) versus control cells (right panel) demonstrating PDGF-BB-induced downregulation of PDGFβ-R mRNA to baseline levels. **b** Effect of IGF-1 addition (left panel) versus control cells (right panel) demonstrating no effect of IGF-1-R on IGF-1-R mRNA expression. Similar results were obtained in three independent experiments.

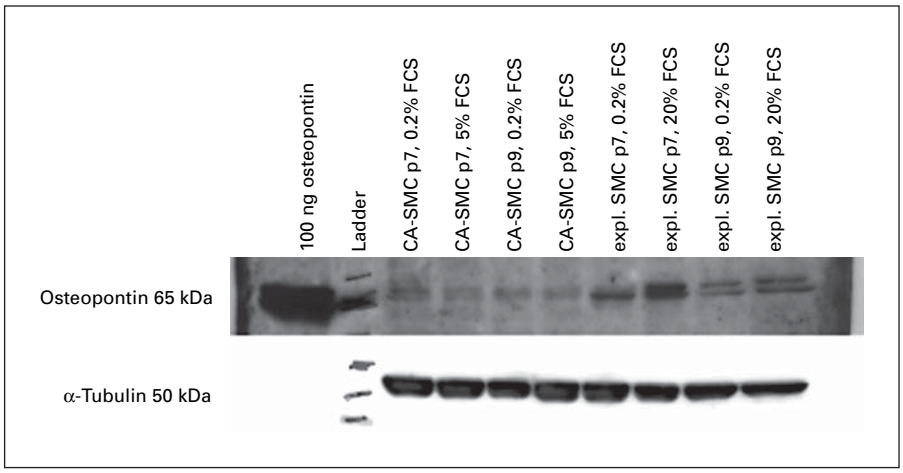

Fig. 4. SMC phenotype classification. Western blot analysis of two independent experiments are shown for CA-SMCs and explanted SMCs, respectively. Explanted SMCs express significantly more osteopontin than CA-SMCs. Serum starvation slightly downregulates osteopontin expression. Similar results were obtained in three independent experiments.

starved SMCs downregulates the starvation-induced increase in PDGFβ-R mRNA expression (fig. 3a, right panel) to baseline levels (fig. 3a, left panel). Serum starvation of SMCs in the presence of PDGF-BB (10 ng/ml) completely abolished the effects of serum starvation on PDGFβ-R mRNA upregulation (data not shown). Corresponding experiments with IGF-1 showed no effect on IGF-1-R expression (fig. 3b).

SMC Phenotype Classification

Osteopontin represents a marker for SMCs of the synthetic phenotype, which are frequently present in human atherosclerotic plaques [33–35]. Western blot analysis for osteopontin was performed with SMCs with and without serum starvation. Osteopontin purchased from SIGMA served as a positive control. Experiments were performed with explanted SMCs and CA-SMCs, respectively. Osteopontin expression was significantly increased in explanted SMCs compared to CA-SMCs. Serum starvation slightly downregulates osteopontin expression in both cell types. Figure 4 shows two representative experiments for both types of SMCs.

SMC Proliferation/Survival

To investigate the effect of serum starvation on SMC proliferation/survival, cells were seeded in 24-well plates and maintained in DMEM/20% FCS (explanted SMCs) or BM/5% FCS (CA-SMCs), respectively. After reaching subconfluence, medium was changed to starvation medium (DMEM/0.2% FCS); after 24 h, total cell numbers were counted, and 20 ng/ml PDGF-BB was added to one population. A second population served as a negative control and was not stimulated with PDGF-BB. In both populations, [^{3}H]-thymidine incorporation was measured 24 h later, and a second cell count was performed after 48 h. Identical experiments were performed with both explanted SMCs and CA-SMCs.

Both, explanted SMCs and CA-SMCs showed a significant increase in [^{3}H]-thymidine incorporation following PDGF-BB stimulation (fig. 5a). Different responses, however, were observed concerning the cell numbers. Whereas in SMCs of the synthetic phenotype (explanted SMCs), PDGF-BB only led to a better cell *survival*, CA-SMCs of the contractile phenotype responded by a significant *proliferation*. Figure 5b shows the changes in total cell numbers for both SMC types.

PDGFβ-R and IGF-1-R Expression in Advanced Human Atherosclerotic Lesions

Figure 6a shows a survey of an advanced human coronary atherosclerotic lesion. The pattern of expression of PDGFβ-R and IGF-1-Rs in advanced human atherosclerotic lesions (n = 5) was analyzed by immunohistochemistry. Figure 6b–e shows a representative experiment of sequential sections of a CA stained for PDGFβ-R, IGF-1-R and α-actin. As depicted in figure 6b, only SMCs in the lamina fibromuscularis [23] express PDGFβ-R, whereas SMCs in the media do not show any expression of this receptor. In contrast, the IGF-1-R is expressed in intimal as well as medial SMCs (fig. 6c), suggesting that expression of this receptor is independent of the location and thus nutritional support [20] of SMCs in the arterial wall. SMCs were identified by staining for α-actin (fig. 6d). Specificity of detected signals was determined by

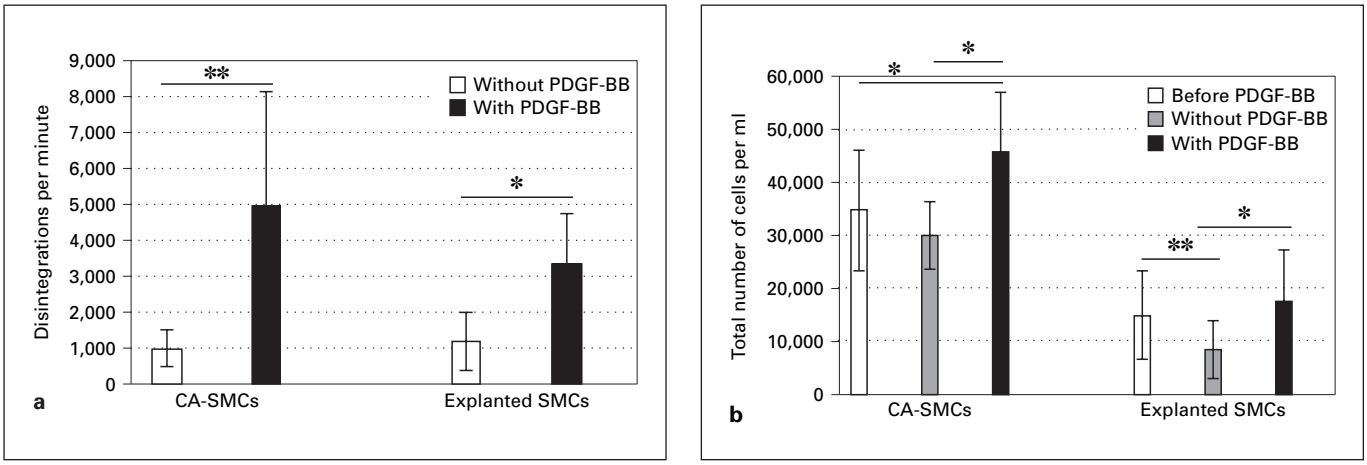

Fig. 5. SMC proliferation/survival. Both explanted SMCs and CA-SMCs showed a highly significant increase in [^{3}H]-thymidine incorporation following PDGF-BB stimulation (**a**). Different responses, however, were observed in the assessment of total cell number (**b**). Whereas in SMCs of the synthetic phenotype (explanted SMCs) PDGF-BB only led to a better cell *survival* but not increased proliferation, CA-SMCs of the contractile phenotype responded by a significant *proliferation*. **a** Paired and unpaired tests. **b** Unpaired test. * significant ($0.05 > p > 0.001$); ** highly significant ($p < 0.001$).

staining with irrelevant isotype-matched IgG (fig. 6e). Immunohistochemistry of healthy parts of the arterial wall showed no PDGFβ-R staining in the lamina fibromuscularis (data not shown).

Discussion

In this study, we demonstrate in an established in vitro model [24, 25] that serum starvation induces upregulation of PDGFβ-R in SMCs in a time-dependent manner, while it had not such effect on IGF-1-R. In turn, PDGF-BB induces downregulation of starvation-induced PDGFβ-R expression whereas IGF-1 does not affect the expression status of its corresponding receptor. Serum-starved SMCs of the synthetic phenotype respond to PDGF-BB by enhanced cell *survival*, whereas SMCs of the contractile phenotype respond by a statistically significant increase in *proliferation*.

Due to different isolation procedures (explant method for explanted SMCs vs. enzymatic digestion for CA-SMCs), explanted SMCs are more likely to be derived from the intima whereas CA-SMCs are more likely to be derived from the media of human CA [26]. Our experiments indeed attributed CA-SMCs to the contractile phenotype (i.e. positive staining for α-actin but only weak detection of osteopontin) whereas explanted SMCs were attributed a synthetic phenotype (i.e. strong detection of osteopontin).

Upregulation of PDGFβ-R by serum starvation may have consequences on atherogenesis on the one hand and restenosis on the other hand: We suggest that in *atherogenesis,* upregulation of PDGFβ-R in SMCs in the thickened lamina fibromuscularis may ensure cell *survival* despite starvation conditions. In *restenosis*, destruction of the endothelial barrier by percutaneous transluminal coronary angioplasty leads to massive influx of plasma components (including PDGF) into the arterial wall and consecutive release of PDGF from platelets. Both mechanisms may contribute to the increase in intimal SMC composition observed in restenosis. In this context, it is interesting to note that the transcription factors c-fos and c-jun, both basically involved in the control of mitosis, are upregulated early after percutaneous transluminal coronary angioplasty. This may in part be due to the activation of the PDGF system, as PDGF is a classical activator of c-fos and c-jun [4, 36].

In either process, atherogenesis or restenosis, the IGF-1 system may play a different role: IGF-1, acting as a progression factor, triggers transition of cells from G1 into S phase [16]. Inhibition of IGF-1-R expression markedly attenuates the effect of other vascular mitogens, especially PDGF and basic fibroblast growth factor, which act as competence factors stimulating the transition of

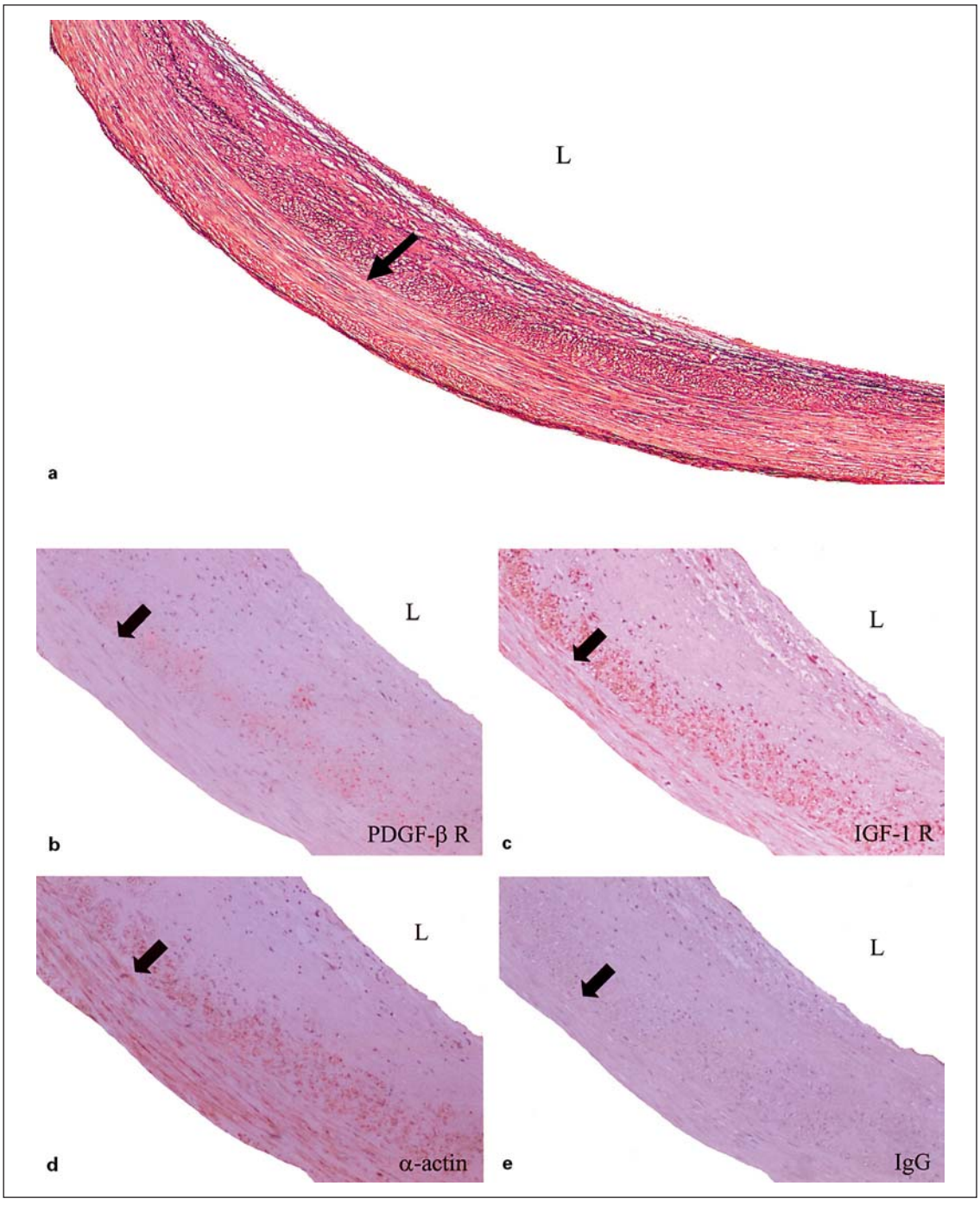

Fig. 6. Growth factor receptor expression in advanced atherosclerotic lesions. **a** Section of an advanced human coronary atherosclerotic lesion presented at low magnification (elastica-van Gieson staining). Lumen (L) is shown on the top. Demarcation between intima and media is indicated by an arrow. ×32, survey image. **b**–**e** Sequential sections of an advanced human coronary atherosclerotic lesion characterized by disorganization of the structure of the intima and changes in the inner contour of the arterial segment. Lumen (L) is on the top. Demarcation between intima and media is indicated by an arrow. ×64. **b** Immunohistochemical staining showing PDGFβ-R-bearing cells in the fibromuscular layer at the base of the intima. Note the lack of PDGFβ-R expression in SMCs in the media. **c** Immunohistochemical staining showing IGF-1-R-bearing cells in the fibromuscular layer at the base of the intima as well as in the media. **d** α-Actin stain identifying PDGFβ-R- and IGF-1-R-bearing cells as being SMCs. **e** Control IgG stain confirming specificity of detected receptor signals.

cells from G0 into G1 phase. Hence, the IGF-1 system seems to translate extrinsic signals into an intrinsic mitogenic response [12, 27, 37]. Lack of dependence of IGF-1-R expression on the location of SMCs in the arterial wall, and thus nutritional supply, may therefore be an additional indicator for the different role of IGF-1 in atherogenesis.

Our in vitro model may reflect in vivo conditions where thickness and thus nutritional support in human arteries might affect the expression of growth factor receptors on human SMCs. We showed by immunohistochemical staining of advanced human coronary atherosclerotic lesions that SMCs in areas of serum starvation, namely the lamina fibromuscularis, express high amounts of PDGFβ-R, whereas these receptors could not be detected in SMCs in the media and in healthy parts of the artery. In contrast, no such differential expression could be observed for IGF-1-R, which is present in SMCs in all areas of the arterial wall. Thus, a link between our in vitro data and ex vivo observations may be offered.

A major limitation of this study is the fact, that our ex vivo data provide a potential link but hardly show any causality. Further experiments are warranted in order to provide mechanistic insights into the transcriptional level and information about growth factor concentrations within atherosclerotic plaques. Furthermore, alternative regulatory mechanisms like oxygen deficit and hypoxia were not investigated.

In conclusion, our data suggest that differential regulation of SMC growth factor receptor expression by serum starvation might be a mechanism for the control of SMC proliferation in atherogenesis and restenosis.

Acknowledgments

We gratefully acknowledge Magdalena Bienek-Ziolkowski and Rosa Herzog for expert technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 451, project A4, given to J.T. and V.H.).

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