Neointimal Smooth Muscle Cells Display a Proinflammatory Phenotype Resulting in Increased Leukocyte Recruitment Mediated by P-Selectin and Chemokines


Abstract—Leukocyte recruitment is crucial for the response to vascular injury in spontaneous and accelerated atherosclerosis. Whereas the mechanisms of leukocyte adhesion to endothelium or matrix-bound platelets have been characterized, less is known about the proadhesive role of smooth muscle cells (SMCs) exposed after endothelial denudation. In laminar flow assays, neointimal rat SMCs (niSMCs) supported a 2.5-fold higher arrest of monocytes and “memory” T lymphocytes than medial SMCs, which was dependent on both P-selectin and VLA-4, as demonstrated by blocking antibodies. The increase in monocyte arrest on niSMCs was triggered by the CXC chemokine GRO-α and fractalkine, whereas “memory” T cell arrest was mediated by stromal cell–derived factor (SDF)-1α. This functional phenotype was paralleled by a constitutively increased mRNA and surface expression of P-selectin and of relevant chemokines in niSMCs, as assessed by real-time PCR and flow cytometry. The increased expression of P-selectin in niSMCs versus medial SMCs was associated with enhanced NF-κB activity, as revealed by immunofluorescence staining for nuclear p65 in vitro. Inhibition of NF-κB by adenoviral IκBα in niSMCs resulted in a marked reduction of increased leukocyte arrest in flow. Furthermore, P-selectin expression by niSMCs in vivo was confirmed in a hypercholesterolemic mouse model of vascular injury by double immunofluorescence and by RT-PCR after laser microdissection. In conclusion, we have identified a NF-κB–mediated proinflammatory phenotype of niSMCs that is characterized by increased P-selectin and chemokine expression and thereby effectively supports leukocyte recruitment.

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Key Words: restenosis ▪ inflammation ▪ cell adhesion molecules ▪ smooth muscle cells ▪ chemokines

The recruitment of leukocytes plays a major role in the response to vascular injury in atherosclerosis and restenosis.1,2 Monocytes are recruited to sites of endothelial denudation and neointimal hyperplasia, eg, after balloon angioplasty, especially in association with hypercholesterolemia.3 Monocyte-derived macrophages contribute significantly to neointimal plaque area and express multiple growth factors, cytokines, and enzymes involved in neointimal growth.1,3 Although the mechanisms of the initial leukocyte adhesion to inflamed endothelium or to matrix-bound platelets emulating the early phase after endothelial denudation have been thoroughly investigated,4 less is known about the proadhesive role of neointimal smooth muscle cells (niSMCs) in mononuclear cell recruitment.

Neointimal SMCs differ from medial SMCs in many ways, eg, in their morphology, proliferative potential, and gene expression, and exhibit a phenotype resembling fetal SMCs.5 Early reports show that modified niSMCs can permanently replace endothelial cells in extensively denuded vessels.6,7 Because neointimal growth is attenuated or even terminated after reendothelialization,8,9 niSMCs exposed to circulating blood could be an important component in the progression of neointimal hyperplasia, eg, by amplifying monocyte recruitment. Activation of NF-κB in niSMCs lining the vessel wall has been found to occur after balloon injury of rat carotid arteries and to be accompanied by enhanced expression of VCAM-1 and monocyte chemotactic protein (MCP)-1, which may mediate monocyte infiltration.9 This may further corroborate the hypothesis that activated niSMCs can form a pseudointima and thus contribute to leukocyte recruitment.

We investigated whether niSMCs differ from medial SMCs in their expression pattern of genes instrumental in monocyte recruitment representing a distinct proinflammatory phenotype and explored the contribution of NF-κB to the regulation of this phenotype. In this study, we show that
niSMCs exhibit a constitutively enhanced expression of P-selectin, CC, and CXC chemokines, and thereby support increased monocyte and "memory" T-cell arrest in flow, resembling the proadhesive phenotype of activated endothelial cells. This phenotype appeared to be attributable to an enhanced constitutive NF-kB activity in niSMCs.

**Materials and Methods**

**Cell Culture and Cell Separation**

SMCs were isolated from media and neointima of adult Sprague-Dawley rats as described. Neointimal cells were obtained by microsopic dissection of the neointimal layer from thoracic aortas 2 weeks after balloon injury. Medial SMCs were obtained from uninjured thoracic aortas. SMCs were grown in Dulbecco’s modified Eagle’s medium-F12 (Life Technologies, containing 10% FCS, penicillin/streptavidin). Experiments were performed between passages 6 and 14; equivalent passages of medial and neointimal cells were compared in each set of experiment. SMC phenotype was confirmed by positive immunostaining for SMC-specific antibodies 6 and 14; equivalent passages of medial and neointimal cells were preincubated with VLA-4 mAb (10 μg/mL, donated by Dr I. Clark-Lewis, University of British Columbia) for 15 minutes at 37 °C. For some experiments SMCs were pretreated with TNF-α (10 μg/mL, PeproTech) overnight. Monolayer or cells were microdissected from the center of neointimal lesions using a laser micromanipulator (PALM MicroBeam). Whole carotid arteries were microdissected from the center of neointimal lesions using a laser micromanipulator (PALM MicroBeam). Whole carotid arteries were subsequently cultured as described.

**Monocyte and CD4+CD45RO+ T-Cell Adhesion on SMCs in Shear Flow**

Laminar flow assays were performed as described. Rat neointimal and medial SMCs were preincubated with or without polyclonal P-selectin antibody (5 μg/mL; cross-reacts with rat P-selectin, Pharmingen), polyclonal fractalkine Ab (20 μg/mL, R&D Systems), polyclonal MCP-1 Ab (20 μg/mL, Pharmingen), or control Abs, for 15 minutes at 37 °C. For some experiments SMCs were preincubated with TNF-α overnight. Monolayer cells or CD4+CD45RO+ T cells (0.5×10^6/mL) in assay buffer (10 mmol/L HEPES, 1 mmol/L Ca^2+Mg^2+, and 0.5% bovine serum albumin) were perfused at 1.5 dyne/cm². For inhibition experiments these cells were preincubated with VLA-4 mAb (10 μg/mL, clone HP1/2), β1-integrin mAb (10 μg/mL, clone TS1/18), CXCR4 mAb (10 μg/mL, clone 12G5, Pharmingen), isotype control, or 8-73GRO-α (1 μg/mL, donated by Dr J. Clark-Lewis, University of British Columbia) at 37 °C for 15 minutes. Firm cell adhesion and rolling (>1 second) after 2 minutes were analyzed in multiple high-power fields recorded by video microscopy.

**RNA Isolation and Real-Time Quantitative RT-PCR Analysis**

Total RNA (2 μg) was isolated from 7×10^6 SMCs (RNEasy, Qiagen) and reverse-transcribed. The following primers were used: β-actin forward, 5'-AGAGGGAAACTGTCGTCGCTAC-3'; reverse, 5'-CGATAGTGATGACCTACGCT-3' (138 bp), P-selectin forward, 5'-CAATAGACCTCTACGCCGAGG-3'; reverse, 5'-CAGTTGACTGCTCCAAAGGTTCCTCG-3' (231 bp); VCAM-1 forward, 5'-GACGATTTACACCTCACTACGCGG-3'; reverse, 5'-CCCTCTAGTAGTCTTCATCTCAGTG-3' (240 bp); GRO-α forward, 5'-GACCCCAAAACCCGAGTCA-3'; reverse, 5'-GCCATGCGCAATCTACTC-3' (146 bp); SDF-1α forward, 5'-GGACGCGCAAGTTGTCGCGCTTG-3'; reverse, 5'-TCGGGTTCAATGCACAC-3' (222 bp).

For quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR), a standard for each primer set was generated by cloning PCR products in pBluescript. A 2-μL cDNA sample was incubated with 25 μl QuantitTeq Mix containing fluorescent dye SYBR Green (Qiagen) and 0.6 μmol/L of each primer pair. PCR amplification was performed after initial denaturation at optimized annealing temperatures for each primer pair using MJ Research Opticon 2 (Biozym). Melting curves were acquired by stepwise increase of the temperature from 55°C to 95°C. Crossing points (Cp) of real-time PCR curves were determined by Opticon Monitor software. The difference of the Cp (ΔCp) of target and β-actin control reflected the amount of target mRNA in each sample. Target mRNA was quantified according to standard curve and normalized to levels of β-actin.

**Flow Cytometry**

For detection of chemokines on the cell surface, confluent medial SMCs and niSMCs were trypsinized, washed, and reacted with fractalkine Ab (R&D Systems), GRO-α Ab (PeproTech), SDF-1α mAb (R&D Systems), or control Abs for 30 minutes at 4 °C. Surface P-selectin was analyzed after fixation with 1% paraformaldehyde (PFA), containing 2% glucose and 0.02% NaN₃, and incubation with polyclonal P-selectin Ab (Pharmingen) or control Ab. Subsequently, cells were stained with fluorescein isothiocyanate (FITC)–conjugated secondary Abs and analyzed by flow cytometry (FACScalibur, BD Biosciences).

**Atherogenic Mouse Model of Restenosis**

Carotid arteries of apolipoprotein E–deficient (apoE−/−) mice (M&B) on high-cholesterol diet were injured as described (n=3). In brief, mice were anesthetized with intraperitoneal ketamine/xylazine and after midline neck incision, the left external carotid artery was tied off distally, and via transverse arteriotomy, a 0.014 in flexible angioplasty guide wire was advanced by 1 cm. Endothelial denudation was achieved by three passes along the common carotid artery with a rotating motion. Two weeks after injury arteries were perfusion-fixed with 4% PFA and embedded in paraffin. Animal experiments were approved by local authorities and complied with German animal protection law.

**Double Immunofluorescence Microscopy**

Carotid artery sections (5 μm, three sections per mouse) were incubated with polyclonal P-selectin Ab (Pharmingen) or an irrelevant rabbit IgG overnight at 4 °C. Antibody binding was detected with a biotinylated secondary Ab and alkaline phosphatase-avidin complex (Vectastain ABC-AP kit) visualized with Vector Red Substrate kit (all from Vector Labs). Subsequently SMA staining was performed with SMA mAb (2 μg/mL, clone 1A4) followed by a FITC-labeled secondary Ab. Incubation with polyclonal p50 Ab (Santa Cruz) for 24 hours at 4 °C was performed after antigen retrieval using a citrate buffer (pH 6, with 0.05% Tween20, 10 minutes in a microwave oven). Ab binding was detected by a biotinylated secondary Ab and avidin-FITC (all Vector labs). Incubation with polyclonal p50 Ab (Santa Cruz) for 24 hours at 4°C was performed after antigen retrieval using a citrate buffer (pH 6, with 0.05% Tween 20, 10 minutes in a microwave oven). Ab binding was detected by a biotinylated secondary Ab and avidin-FITC (all Vector labs). Finally, sections were mounted and analyzed by fluorescence microscopy (Leica DMLB microscope).

SMCs were cultured on chamber slides (Falcon) until confluence. After methanol fixation (5 minutes, −30 °C) double immunofluorescence staining for P-selectin (polyclonal P-selectin Ab, Pharmingen) and SMA (1A4, DAKO) was performed essentially as described earlier. Combined p65 and P-selectin immunostaining was performed with a polyclonal p65 Ab (Santa Cruz) for 1 hour, reacted with a biotinylated secondary Ab and avidin-FITC. Subsequently staining for P-selectin was performed with a polyclonal P-selectin Ab (Santa Cruz), detected by a biotinylated secondary Ab and avidin-TRITC (Sigma).

**Laser Microdissection and PCR Analysis**

Microdissection of neointimal tissue was performed on 5-μm-thick paraffin-embedded sections of the common carotid artery of apoE−/− mice 2 weeks after wire injury. After immunostaining for α-SMA (clone 1A4) and counterstaining with hematoxylin, α-SMA–positive cells were microdissected from the center of neointimal lesions using a laser micromanipulator (PALM MicroBeam). Whole carotid arteries were used as controls. Microdissected cells were directly transferred into reaction tubes containing RNA stabilization reagent (RNAlater, Qiagen), and RNA was isolated (RNAsley Micro kit, Qiagen) and treated with DNase I.
Proinflammatory Phenotype of Neointimal SMCs

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Overexpression of Adenoviral-Encoded IκB-β

Construction of the adenoviral vector encoding for IκB-β (rAD.IκB-β; a kind gift from Dr R. de Martin, Vienna International Research Center, Austria) and adenoviral gene transfer were performed as described.15 Briefly, subconfluent SMCs were washed with PBS and incubated at a multiplicity of infection (MOI) of 500 (plaque forming units, PFU) with rAD.IκB-β or control adenovirus encoding the β-Galactosidase protein (rAD.lacZ) in medium without FCS. After 1 hour at 37°C, the adenovirus was washed off and fresh growth medium was added. To analyze the efficiency of transduction, rAD.lacZ-infected SMCs were stained with X-Gal after 48 hours and the percentage of β-Gal–positive SMCs was determined by bright-field microscopy (60.5±8.7%, n=4).

Gel Shift Analysis

Nuclear protein (10 μg) was isolated from untreated niSMCs or from niSMCs 24 hours after adenoviral gene transfer as previously described.13 After incubation for 15 minutes with [32P]-dATP-labeled, double-stranded oligonucleotides corresponding to the NF-κB binding motif 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (Santa Cruz) for 15 minutes, samples were separated on nondenaturing 4% polyacrylamide gels and exposed to x-ray films.

Statistical Analysis

Data are expressed as mean±SD unless otherwise stated. Statistical analysis was performed with Prism Software (GraphPad) by use of two-tailed Student’s t test or one-way ANOVA and Newman-Keuls post test. Differences with P<0.05 were considered as statistically significant.

Results

Medial SMCs have been shown to support efficient monocyte adhesion in stasis only when stimulated with cytokines or modified lipoproteins.16 Less is known about the adhesive properties of niSMCs, which display a phenotype distinct from medial SMCs and become exposed to circulating leukocytes after endothelial denudation. Hence, we compared the propensity of niSMCs and medial SMCs without cytokine stimulation to recruit monocytes under flow conditions. Rolling of monocytes as a prerequisite for firm arrest in the adhesion cascade17 was observed on niSMCs, but was barely detectable on medial SMCs (Figure 1A). The shear-resistant arrest of monocytes on niSMCs was 2.5-fold higher than that supported by resting medial SMCs (Figure 1B). Stimulation with TNF-α increased monocyte arrest on both neointimal and medial SMCs to almost equal levels (data not shown).

We next explored whether adhesion molecules involved in monocyte recruitment on endothelium also participate in monocyte arrest on SMCs. Pretreatment of SMCs with a blocking P-selectin Ab markedly inhibited monocyte rolling and arrest on niSMCs but did not affect adhesive interactions on medial SMCs (Figures 1A and 1B). Blocking VLA-4, the monocytic receptor for VCAM-1, almost completely abolished monocyte rolling and arrest on niSMCs and medial SMCs (Figures 1A and 1B). In contrast, blocking monocyte β3-integrins had no effect (data not shown). Thus, monocyte rolling and arrest on SMCs is generally dependent on VLA-4, whereas the substantial enhancement of monocyte arrest on niSMCs is mediated by P-selectin. Chemokines and their receptors are crucial for triggering integrin-dependent arrest of monocytes on inflamed or atherosclerotic endothelium.11,18 Accordingly, monocyte arrest on niSMCs was significantly decreased by pretreatment of monocytes with the GRO-α/KC receptor CXCR2 antagonist 8,9-GRO-α (by almost 50%) or by pretreatment of niSMCs with a blocking mAb to fractalkine (Figure 1C).

In contrast, blocking MCP-1 or the SDF-1α receptor CXCR4 on monocytes did not significantly affect monocyte arrest (Figure 1C). Thus, the CXCR2 and fractalkine rather than MCP-1 and SDF-1α appear to be involved in triggering VLA-4–mediated monocyte arrest on niSMCs.

A role of CD4+CD45RO+ memory T cells in the formation of atherosclerotic plaques has been described.19 Hence, we tested whether niSMCs can also recruit memory T cells in flow. Compared with medial SMCs, niSMCs supported a significantly enhanced arrest of CD4+CD45RO+ T cells, which was inhibited by blocking T-cell VLA-4 (Figure 1D). Unlike monocyte arrest on niSMCs, enhanced VLA-4–mediated memory T-cell arrest could be abolished by pretreatment with the CXCR4 mAb (Figure 1D), indicating that it was triggered by SDF-1α.

To provide a molecular basis for the remarkable difference in the adhesive phenotype between the distinct SMC subtypes, we next investigated the expression of relevant adhesion molecules and chemokines by quantitative real-time RT-PCR (Figure 2 and online Figure 1) available in the online data supplement at http://circres.ahajournals.org. In line with the functional differences, the expression of P-selectin mRNA was hardly detectable in medial SMCs, but substantially upregulated (>2.5-fold) in niSMCs (Figure 2). Accordingly, the expression of GRO-α and SDF-1α transcripts was 3-fold and 2-fold higher in niSMCs compared with medial SMCs (Figure 2). In contrast, differences in VCAM-1 expression between niSMCs and medial SMCs were negligible (Figure 2), indicating that the differential expression of P-selectin and chemokines was sufficient to explain differences in VLA-4–mediated arrest. In extension to mRNA expression analysis, we studied surface expression of the functionally relevant proteins by flow cytometry. Surface expression of P-selectin was restricted to a SMC subset (Figures 2E and 3A), which was expanded among niSMCs (65±15%, n=3, Figure 3E) compared with medial SMCs (30±7%, n=3, Figure 3A). Furthermore, surface P-selectin was increased on the P-selectin–positive subset of niSMCs (Figure 3E). Double immunofluorescence staining for P-selectin and α-SMA confirmed expression of P-selectin on niSMCs and medial SMCs (online Figure 2). The surface expression of fractalkine was slightly enhanced on niSMCs (Figure 3F) compared with medial SMCs (Figure 3B). The presence of immobilized GRO-α (Figures 3C and 3G and online Figure 3) and SDF-1–α (Figures 3D and 3H) on the cell surface, a prerequisite for their role as arrest chemokines, was not evident in medial SMCs (Figures 3C and 3D), but clearly detectable on niSMCs (Figures 3G and 3H). In contrast to GRO-α and SDF-1–α, the CC chemokine MCP-1 was not
immobilized on the surface of SMCs, albeit upregulation of MCP-1 in niSMCs was found by ELISA of SMC lysates (online Figure 4). Thus, the functional differences between SMC phenotypes were attributable to changes in their gene expression profile, which account for increased surface expression of proteins relevant for leukocyte arrest.

In accordance with the notion that the induction of genes, such as GRO-α and P-selectin, involves the transcription factor NF-κB, a gene array analysis confirmed that in addition to cell cycle–associated genes, a cluster of NF-κB–dependent transcripts for chemokines and adhesion molecules was upregulated in niSMCs versus media SMCs (U. Zeiffer et al, unpublished data, 2004). This prompted us to analyze whether NF-κB was also involved in establishing the neointimal SMC phenotype. Nuclear translocation of the Rel family protein p65 as a marker for NF-κB activity was determined by immunofluorescence staining in vitro. Compared with medial SMCs where p65 was rarely detected in the nucleus (3 ± 1.3% n = 7, Figure 4A), the proportion of niSMCs with nuclear p65 was substantial (35 ± 4%, n = 11, Figure 4A). Furthermore P-selectin expression was exclusively evident in niSMCs with nuclear p65, as determined by combined immunofluorescence staining, suggesting a correlation of NF-κB activity and P-selectin expression (Figure 4B).

To ultimately demonstrate the direct contribution of the enhanced constitutive NF-κB activity to the niSMC phenotype, adenovirus-mediated transfer of the NF-κB inhibitor IkB-α was performed. Electrophoretic mobility shift assays demonstrated DNA binding of NF-κB after gene transfer with the control adenovirus rAD.lacZ (Figure 5A), which was
Figure 2. Differential mRNA expression of P-selectin, GRO-\(\alpha\), and SDF-1\(\alpha\) in neointimal and medial SMCs as assessed by quantitative real-time PCR. P-selectin, GRO-\(\alpha\), and SDF-1\(\alpha\) mRNA expression normalized to \(\beta\)-actin is significantly increased in niSMCs, whereas VCAM-1 is not differentially expressed. Data are mean \pm SD from at least 3 independent experiments. \(* P<0.05.\)

Figure 3. Flow cytometry analysis of P-selectin (A and E), fractalkine (B and F), GRO-\(\alpha\) (C and G), and SDF-1\(\alpha\) (D and H) surface expression levels on SMCs. Representative histogram plots of control Ab staining (filled) and antigen-specific Ab (bold) of medial SMCs (top) and niSMCs (bottom) are shown. P-selectin surface expression on medial (A) and neointimal SMCs (E) is restricted to a subset of SMCs, which was expanded among neointimal SMCs. Enhanced surface expression of fractalkine (B and F), GRO-\(\alpha\) (C and G), and SDF-1\(\alpha\) (D and H) were detected on niSMCs compared with medial SMCs.

Figure 4. A, Immunofluorescence staining for p65 in niSMCs and medial SMCs in vitro. Nuclear translocation of p65 was rarely evident in medial SMCs, whereas a significant population of niSMCs revealed nuclear p65 staining (indicated by asterisks). Scale bars = 50 \(\mu\)m. B, Combined immunofluorescence staining for p65 (green) and P-selectin (red) demonstrates P-selectin expression predominantly in niSMCs with nuclear p65 staining. Scale bar = 20 \(\mu\)m.

In DNA binding of NF-\(\kappa\)B by I\(\kappa\)B-\(\alpha\) overexpression was associated with a marked reduction of the increased arrest of monocytes (Figure 5B) and CD4\(^+\) CD45RO\(^+\) T cells (Figure 5C) on niSMCs.

To evaluate the role of niSMCs expressing P-selectin in vivo, neointimal lesions of apoE\(^{-/-}\) mice 2 weeks after carotid wire injury were studied by immunofluorescence staining for \(\alpha\)-SMA and P-selectin. In neointimal sections where reendothelialization was established, P-selectin--expressing SMCs were regularly found in deeper neointimal layers (Figure 6A). It has been described that up to 25% of the denuded area is not covered by endothelial cells 2 weeks after carotid wire injury.\(^{21}\) In this neointimal area devoid of endothelium, P-selectin--expressing SMCs were detected at the luminal lining, a pseudoendothelium (Figure 6B). To verify the transcriptional upregulation of P-selectin in niSMCs, RT-PCR analysis was performed in microdissected \(\alpha\)-SMA–positive cells derived from neointimal lesions. Although \(\alpha\)-SMA staining ensured positive identification of niSMCs, VE-cadherin mRNA expression was analyzed as a marker for potential contamination of the samples with endothelial cells. In \(\alpha\)-SMA\(^+\)/VE-cadherin\(^-\) neointimal cells, P-selectin expression was clearly evident (Figure 6C), thereby identifying neointimal SMCs as a source of P-selectin in vivo. Finally, immunofluorescence staining for p50 in vivo revealed nuclear translocation of p50 in the majority of neointimal cells, including luminal cells, with a distribution resembling P-selectin expression 2 weeks after wire-induced injury (Figure 6D). Solely cytoplasmic p50 was detectable in sections from uninjured carotid arteries (online Figure 5). This further implicates enhanced NF-\(\kappa\)B activity as a crucial regulator controlling the niSMC phenotype.

Discussion

In this study we have found that cultured niSMCs display a proadhesive phenotype that is characterized by increased leukocyte recruitment and associated with a constitutive upregulation of P-selectin and the chemokines GRO-\(\alpha\), SDF-1\(\alpha\), and fractalkine. P-selectin and GRO-\(\alpha\) markedly enhanced VLA-4–dependent monocyte adhesion to niSMCs under flow conditions, whereas the SDF-1\(\alpha\) receptor CXCR4 selectively mediated the arrest of memory T cells via VLA-4. In addition, the pronounced surface expression of fractalkine on niSMCs supported monocyte recruitment, conceivably via...
integrin-independent mechanisms. This proinflammatory phenotype appears to be the functional consequence of enhanced constitutive NF-κB activity in nSMCs, as indicated by the reduction of increased leukocyte arrest after inhibition of NF-κB activity. Upregulation of P-selectin expression was confined to a subset of nSMCs, in which P-selectin was diffusely distributed on the cell surface. In vivo, SMCs expressing P-selectin and SMCs exhibiting constitutive NF-κB activity could be found in deeper neointimal layers, as well as contributing to the luminal lining of neointimal lesions 2 weeks after arterial injury in apoE−/− mice. Studies in apoE−/− mice have shown that the absence of P-selectin reduces aortic lesions by 40% to 62%, an effect that is attributable to diminished macrophage infiltration. Indeed, blocking P-selectin inhibits monocyte rolling and adhesion on early atherosclerotic lesions in carotid arteries of apoE−/− mice. After wire-induced endothelial denudation in apoE−/− mice, neointima formation and macrophage recruitment were almost completely prevented in the absence of P-selectin, revealing an even more substantial involvement of P-selectin. In contrast to spontaneous atherosclerosis, the expression of P-selectin has been detected in media and developing neointima after carotid ligation, but its functional implications have not been elucidated. Because endothelial denudation with subsequent neointima formation results in the exposure of nSMCs to the blood stream before reendothelialization is accomplished, P-selectin on nSMCs may serve to support monocyte recruitment. We could confirm an upregulation of P-selectin in nSMCs compared with uninjured medial SMCs. The enhanced expression of P-selectin was involved in increased monocyte rolling and arrest on nSMCs in flow, by serving as a prerequisite for firm arrest via VLA-4, by triggering VLA-4 avidity alone, or in synergy with chemokines. Because P-selectin–expressing SMCs were identified along the luminal lining and in directly subluminal portions of the arterial neointima, this may represent a SMC phenotype with the propensity to establish a pseudoendothelium after denudation.

Like endothelial cells, vascular SMCs can become a potent source for different chemokines, eg, MCP-1 or interleukin-8 on stimulation with inflammatory cytokines. Chemokines and their receptors, which can exhibit specialized functions in the multistep process of leukocyte recruitment, have been implicated in the progression of atherosclerosis and developing neointima after carotid ligation.

Figure 5. Effect of IκB-α overexpression in nSMCs. A, Electromobility shift assays were performed to detect NF-κB DNA binding activity in nSMCs after adenoviral transfer of the lacZ reporter gene (rAD.lacZ) or the IκB-α gene (rAD.IκB-α). Autoradiography of 4 independent samples per group is shown. Monocyte (B) and CD4+CD45RO+ T cells (C) arrest on nSMCs after infection with rAD.IκB-α or control virus (rAD.lacZ) was determined under flow conditions. Number of adherent cells was expressed as the percentage of that in untreated controls. Data are mean±SD from at least 3 independent experiments. *P<0.05.
restenosis. Interestingly, we found that various chemokines involved in atherogenic monocyte recruitment were constitutively upregulated in niSMCs. For instance, GRO-α, which triggers VLA-4–dependent monocyte adhesion to early atherosclerotic lesions, is expressed and immobilized on the surface of niSMCs and stimulates VLA-4–mediated monocyte arrest on niSMCs in flow. Conversely, the enhanced expression of SDF-1α in niSMCs did not contribute to monocyte arrest, which is in accordance to in vivo results in apoE−/− mice after carotid wire-injury, demonstrating that treatment with a neutralizing antibody to SDF-1α had no effect on neointimal macrophage content. On the other hand, the dramatic increase in VLA-4–dependent arrest of proatherogenic CD4+CD45RO+ T cells involved SDF-1α, as shown by blocking its receptor CXCR4. This finding suggests that different SMC-derived chemokines cooperate in the regulation of subtype-specific leukocyte recruitment. Furthermore, surface expression of fractalkine was upregulated on niSMCs and a neutralizing fractalkine Ab reduced monocyte arrest under flow conditions. Fractalkine can mediate mono-
cyte adhesion to endothelial cells in an integrin-independent fashion and therefore may participate in monocyte recruitment on niSMCs by a similar and distinctive mechanism. In apoE−/− mice, lesional SMCs express fractalkine, particularly in the vicinity of macrophages, implying a role of SMC-derived fractalkine in macrophage infiltration in atherosclerosis. Our data indicate that this role may extend to neointimal monocyte recruitment after endothelial denudation.

The proadhesive phenotype of niSMCs was accompanied by an increased constitutive nuclear translocation of p50/p65 proteins. In contrast to endothelial cells, where NF-κB is transiently activated by cytokines, a constitutive and sustained NF-κB activation has been described in SMCs in vitro and in vivo, presumably due to a divergent NF-κB regulation. Furthermore, NF-κB DNA binding activity in SMCs was increased up to 14 days after arterial injury in a rat model, concurrently with monocyte adherence to luminal niSMCs. Because the transcription of multiple inflammatory genes upregulated in niSMCs, eg, P-selectin, GRO-α, MCP-1, and fractalkine, is controlled by NF-κB, this transcription factor might integrate the intracellular signals finally leading to neointimal leukocyte recruitment. The finding that inhibition of NF-κB activity by overexpression of IκB-α diminished leukocyte arrest in vitro supports the concept that NF-κB is centrally involved in generating the proadhesive phenotype of niSMCs. This hypothesis is further substantiated by the correlation of P-selectin expression and NF-κB activity in vitro and in vivo. Surprisingly, the expression of VCAM-1 was comparable in niSMCs and medial SMCs. This is consistent with findings that VCAM-1 gene expression during differentiation of cultured SMCs was independent of NF-κB and that the upregulation of neointimal expression after arterial injury in vivo was transient for VCAM-1 and prolonged for MCP-1, indicating a dissociation of their regulation. The sustained NF-κB activation and conversion into a proadhesive phenotype may occur either through persistence of stimulating agents, eg, by autocrine production of growth factors or cytokines, or through elements modifying the activation of the NF-κB signaling cascade, such as the IκB kinase activity.

The present characterization of a proadhesive phenotype with its molecular basis in niSMCs provides novel insights into their pathophysiological role, which may comprise the adoption of endothelial-like functions in luminal cell lining and inflammatory leukocyte recruitment during neointima formation. This may advance the identification of molecular targets selectively directed at niSMCs and their functions in the prevention of restenosis.

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Neointimal smooth muscle cells display a proinflammatory phenotype resulting in increased leukocyte recruitment mediated by P-selectin and chemokines


Supplemental Figure 1: Differential mRNA expression of P-selectin, GRO-α and SDF-1α in neointimal and medial SMCs. Real-time RT-PCR curves for 4 different genes (P-selectin, GRO-α, SDF-1α and VCAM-1) of representative niSMC (A, left row) and medial SMC (A, right row) samples with respective β-actin controls are shown. The differences of the CPs (ΔCP) of target and β-actin are specified. A smaller ΔCP indicates a higher amount of target mRNA.
Supplemental Figure 2: P-selectin is up-regulated and redistributed on the cell surface in a subset of niSMCs. Double immunofluorescence staining of P-selectin and α-SMA reveals a faint and perinuclear P-selectin staining in a small subset of P-selectin⁺-medial SMCs (A), whereas P-selectin on niSMCs was more uniformly distributed on the cell surface excluding the nuclear region (B). Scale bars, 20 μm.

Supplemental Figure 3: Immunofluorescence microscopy of GRO-α reveals abundant deposition of GRO-α on niSMCs (A) but not on medial SMCs (B). Non-permeabilized cells were stained for surface GRO-α after fixation with 4 % PFA (8 min at room temperature) using a polyclonal anti-rat GRO-α Ab (abcam.com) and a FITC-labeled secondary Ab. Scale bars, 20 μm.
Supplemental Figure 4: (A) Protein content of MCP-1 in lysates from medial and niSMCs. Cells were homogenized in RIPA-buffer (with 1 % Triton X-100, 0.1 % SDS, 1 % deoxycholic acid, 1 mmol/L PMSF, 5 µg/ml aprotinin and 5 µg/ml leupeptin) and total protein (DC Protein Assay, Biorad) as well as MCP-1 protein (Mouse JE/MCP-1 Quantikine ELISA, crossreacts with rat MCP-1, R&D Systems) concentration was determined. MCP-1 levels were normalized to total protein concentration and expressed as n-fold increase compared to medial SMCs. *p<0.05, mean±SEM, n=3; (B) Surface expression of MCP-1 on medial (filled histogram) and neointimal SMCs (bold line) was evaluated by FACS analysis. After trypsin treatment cells were incubated with a phycoerythrin-conjugated MCP-1 Ab (2H5, BD Pharmingen) for 30 min at 4°C.

Supplemental Figure 5: Immunofluorescence staining for p50, performed as described in the Materials and Methods section, in uninjured carotid arteries from apoE−/− mice revealed exclusively cytoplasmic p50. Nuclei were counterstained with DAPI (4',6 diamidino-2-phenylindole). Scale bars, 20µm.
Supplemental Figure 6: Immunofluorescence staining for α-SMA in neointimal lesions after wire injury of the carotid artery in apoE⁻/⁻ mice. After blocking of unspecific protein binding with 5% bovine serum albumin, sections were incubated with α-SMA mAb (2 µg/ml, clone 1A4, Dako) or isotype control Ab (2 µg/ml) for 1 h at room temperature. Binding of the primary antibody was detected by a FITC-conjugated anti-mouse Ab (Sigma). Isotype control treated sections reveal only negligible background staining. Scale bar, 100 µm.