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SMC-Specific IGF-1 Overexpression in *Apoe*$^{-/-}$ Mice Does Not Alter Atherosclerotic Plaque Burden but Increases Features of Plaque Stability

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Abstract

Objective—Growth factors may play a permissive role in atherosclerosis initiation and progression, in part via their promotion of VSMC accumulation in plaques. However, unstable human plaques often have a relative paucity of VSMC which has been suggested to contribute to plaque rupture and/or erosion and to clinical events. IGF-1 is an endocrine and autocrine/paracrine growth factor that is a mitogen for VSMC, but when infused into *Apoe*$^{-/-}$ mice paradoxically reduces atherosclerosis burden.

Methods & Results—To determine the effect of stimulation of VSMC growth on atherosclerotic plaque development and to understand mechanisms of IGF-1's atheroprotective effect we assessed atherosclerotic plaques in mice overexpressing IGF-1 in SMC under the control of the αSMA promoter, after backcrossing to the *Apoe*$^{-/-}$ background (SMP8/*Apoe*$^{-/-}$). When compared with *Apoe*$^{-/-}$ mice these SMP8/*Apoe*$^{-/-}$ mice developed comparable plaque burden after 12 wks on a Western diet, suggesting that the ability of increased circulating IGF-1 to reduce plaque burden was mediated in large part via non-SMC target cells. However, advanced plaques in SMP8/*Apoe*$^{-/-}$ mice displayed several features of plaque stability, including increased fibrous cap area, αSMA positive SMC and collagen content and reduced necrotic cores.

Conclusion—These findings indicate that stimulation of VSMC IGF-1 signaling does not alter total atherosclerotic plaque burden and may improve atherosclerotic plaque stability.

Atherosclerosis is the leading cause of mortality in the western world[1] and is characterized by progression of vascular lesions from early fatty streaks to more advanced plaques characterized by arterial intimal thickening, inflammatory cell and vascular smooth muscle cell (VSMC) accumulation, extra-cellular lipid (atheroma) and fibrous tissue deposition[2, 3]. While migration, proliferation and matrix deposition of VSMC contributes to lesion development[4], it is unclear whether VSMC accumulation is linked to clinical events. In fact, acute coronary events result more often from erosion or rupture of unstable plaques that have large lipid pools, are rich in lipid-laden macrophages and have thin fibrous caps reflecting a relative reduction in VSMC number.[4, 5] Thus, increased intimal accumulation of VSMC could contribute to atherosclerotic plaque stabilization.

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Traditionally the role of growth factors in atherosclerosis has been thought to be permissive and to promote neointima formation.[6–8] IGF-1 is an endocrine and autocrine/paracrine growth factor that exerts pleiotropic effects on cells involved in atherogenesis.[9] Notably, it has mitogenic and antiapoptotic actions on endothelial cells and VSMC and stimulates VSMC migration.[10] Consistent with the role of IGF-1 as a VSMC mitogen most[11–14] but not all[15] studies using inhibitors have demonstrated that reduced VSMC IGF-1 signaling correlates with decreased neointimal responses to mechanical arterial injury. Furthermore, targeted overexpression of IGF-1 in SMC increases neointimal formation.[16] However, while mechanical arterial injury models provide much information about the restenotic process they have significant limitations when addressing mechanisms of atherogenesis and the role of VSMC IGF-1 in the latter process remains unclear.

We have previously shown that oxidized LDL downregulates IGF-1 and IGF-1 receptor expression in vascular smooth muscle cells[17, 18] and that expression of IGF-1 and IGF-1 receptor is reduced in areas of advanced human plaque staining positive for oxidized LDL.[19] These findings suggested that decreased IGF-1 activity could contribute to the atherosclerotic process and notably to depletion of VSMC in advanced unstable plaque. Similar findings were reported in cultured plaque-derived VSMC.[20] We recently reported that infusion of recombinant human IGF-1 into *Apoe*-deficient mice on a western diet for 12 wks reduced systemic and vascular oxidant stress and vascular pro-inflammatory cytokine expression, and delayed atherosclerosis progression.[21] These unexpected atheroprotective effects of an increase in circulating IGF-1 raised many questions including that of the target cells involved. We designed the present study to determine the role of VSMC IGF-1 in atherogenesis by crossing SMP8-IGF-1 transgenic mice that overexpress a rat IGF-1 transgene under the control of the α-smooth muscle actin (αSMA) gene promoter [22] into *Apoe*$^{-/-}$ mice. We report that contrary to what could be expected from results of mechanical injury models overexpression of IGF-1 in VSMC did not alter atherosclerotic plaque burden but increased atherosclerotic plaque VSMC and collagen content and fibrous cap thickness. Our findings suggest that autocrine VSMC IGF-1 signaling promotes atherosclerotic plaque stability.

Methods

Materials

IGF-1 ELISA kits were obtained from Diagnostic Systems Laboratories (DSL) (Webster, TX). Dihydroethidium (DHE) was from Invitrogen (Carlsbad, CA). Oil Red-O was from Biomedicals Inc. (Irvine, CA). Antibodies used were: anti-α-smooth muscle actin, anti-calponin, and anti-SM22α from Abcam, anti-smooth muscle myosin heavy chain from Millipore, anti-collagen type I and III from Rockland Immunochemicals. Polyethylene glycol-superoxide dismutase (PEG-SOD) was from Sigma-Aldrich.

Animals

All animal experiments were performed according to protocols approved by the IACUC. SMP8-IGF-1 transgenic mice (in FVB background) were obtained from Dr. James A. Fagin, University of Cincinnati, Cincinnati, Ohio.[22] SMP8-IGF-1 transgene positive mice (SMP8) were identified by PCR and bred to *Apoe* knockout (*Apoe*$^{-/-}$) (C57BL/6 background) mice for 8 generations before using them for experiments. Twelve SMP8/*Apoe*$^{-/-}$ and 24 *Apoe*$^{-/-}$ mice 8 wks of age were fed with a western type diet (42% of total calories from fat; 0.15% cholesterol, Harlan, Indianapolis, IN) for 12 wks, and 6 SMP8/*Apoe*$^{-/-}$ and 6 *Apoe*$^{-/-}$ mice 8 wks of age were fed with a western type diet for 4 wks.

Atherosclerosis Quantification

Atherosclerosis burden was quantified by measuring the surface area of Oil Red O positive lesions on *en face* preparations of whole aortas as previously described.[21] Additionally, serial sections (6 μm) were taken throughout the entire aortic valve area as per Paigen et al.,[23] and stained with H&E for quantitation of plaque cross-sectional area as previously described.[21] The necrotic core was identified as part of the atherosclerotic plaque which appeared to be acellular (hematoxilin-negative) and often contained cholesterol crystals. SMC fibrous cap area was measured using αSMA stained aortic valve sections with the cap identified as the immunopositive part of the plaque covering the necrotic core. Necrotic core and fibrous cap area were measured using Image-Pro and data presented as percent total plaque area.

Immunohistochemical Analysis

Serial 6-μm paraffin-embedded cross sections were taken throughout the entire aortic valve area, and 3 sets of serial sections obtained at 60-μm intervals were used for measurement of macrophage positive lesion area using rat anti-mouse Mac-3 monoclonal antibody as previously described.[21] Serial sections were also stained for TNF-α, αSMA and calponin. Antibodies for immunohistochemistry were purchased from BD Pharmingen (rat anti-mouse Mac-3 antibody), Chemicon/Millipore (mouse α-smooth muscle actin antibody), Fitzgerald Industries (rabbit TNF-α antibody) and Abcam (rabbit Ki67 and rabbit anti-mouse calponin antibodies). 4′,6-diamidino-2-phenylindole (DAPI) was purchased from Invitrogen. Sections were also stained with Trichrome (Richard-Allan Scientific, Kalamazoo, MI) and with picrosirius red (Polysciences Inc, Warrington, PA). Picosirius red stained sections were imaged with a light microscope (Olympus) and also examined under polarized light with a Motic BA300 Pol polarized microscope.[24]

Aortic Superoxide Measurement

Aortic superoxide levels were measured with DHE on serial frozen sections (10 μm) obtained from the aortic root, as previously described.[21] DHE fluorescence was quantified by averaging the mean fluorescence intensity within 3 identical circles placed on a plaque and adjacent media using Image-Pro and expressed as PEG-SOD-inhibitable fluorescence after subtraction of the DHE signal obtained from scavenger-pretreated section. Three to 5 sections from each animal were analyzed.

Quantitative Real-Time RT-PCR

Total RNA extraction and real-time PCR was performed as previously described.[25] Briefly, total aortic RNA was isolated using the TriPure Isolation Reagent (Roche) followed by purification with the RNeasy mini kit (Qiagen). cDNA was synthesized using the First Strand cDNA Synthesis kit (Amersham) and used for the 40-cycle 2-step PCR with sequence-specific primer pairs in the iCycler IQ Real-Time Detection System (Bio-Rad). Primer sequences are provided in supplemental data.

Quantification of Cell Apoptosis and Cell Proliferation

Cell apoptosis was quantified in paraffin-embedded aortic valve cross sections with the Apoptosis TUNEL detection kit (Fluorescein) from Roche as per manufacturer's instructions. To identify apoptotic VSMC in the atherosclerotic plaque, TUNEL-stained sections were co-stained with αSMA antibody (1:1000) followed by incubation with biotinylated secondary antibody and streptavidin-Alexa 594 complex (Invitrogen). Sections were mounted with DAPI-contained mounting media (Vector Laboratories Inc). Total cell apoptosis was defined as TUNEL-positive cell number per 1000 plaque cells and SMC apoptosis was measured as the number of αSMA/TUNEL-double positive cells per 1000 αSMA-positive cells.

To assess cell proliferation, in vivo labeling of DNA with 5-ethynil-2′-deoxyuridine (EdU) was performed according to Salic et al [26]. EdU (5gm//kg/day) was administered i.p. for 4 d before sacrifice. The aorta was perfuse-fixed, paraffin embedded, and 5 μm cross-sections processed for detection of labeled DNA using Alexa Fluor 488- azide (Clidk-iT Imaging Kit, Invitrogen).

Cell culture

Cultured human aortic smooth muscle cells (HASMC, Lonza) were grown in SmBM medium (Lonza) supplemented with 5% fetal calf serum, antibiotics, human recombinant Epidermal Growth Factor, Insulin, and human recombinant Fibroblast Growth Factor. The cells were used for experiment at passage 4 to 10. All the experiments were done in serum free condition using 1:1 mixture of Dulbecco's modified essential medium and F-12 nutrient solution (Gibco).

Western blot analysis

Western blot analysis was performed as described previously [27]. In brief, cells were washed with PBS and lysed in RIPA buffer, containing 150 mM NaCl, 20 mM Tris-Cl, pH 7.2, 1 mM EDTA, 1% NP40, 5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 0.1 M okadaic acid, 0.1 μM aprotinin, 10μg/ml leupeptin, and 10 mM NaF. Lysates were subjected to 10% SDS-PAGE and western blotting analysis. Immunopositive bands were visualized by enhanced chemiluminescence (ECL, Amersham). Blots were stripped and reprobed with monoclonal anti-β-actin antibody as a control for equal loading.

Statistical Analysis

All numerical data are expressed as mean ± SEM. Two-tailed unpaired Student *t* tests were performed to determine statistical significance. Differences were considered significant at $P<0.05$.

Results

SMC Targeted Overexpression of IGF-1 in *Apoe*$^{-/-}$ mice did not change body weight, circulating IGF-1 levels, blood pressure or total cholesterol

The mean body weight and systolic blood pressure of SMP8/*Apoe*$^{-/-}$ mice was not different than *Apoe*$^{-/-}$ over the duration of 12 wks on a western diet (Fig 1A, B). Likewise, circulating IGF-1 levels were not different between *Apoe*$^{-/-}$ or SMP8/*Apoe*$^{-/-}$ mice, either at 8 wks old or after 12 wks on a western diet (Fig 1C), consistent with the original report from Wang et al[22], indicating that overexpression of IGF-1 in SMC did not raise circulating IGF-1[22]. However, interestingly, after 12 wks on this diet both SMP8/*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice had IGF-1 levels that were increased (by 67% and 56% respectively) compared to baseline IGF-1 levels prior to diet initiation (Fig 1C). To confirm that the increase in IGF-1 levels was not age-related we performed an additional experiment and measured IGF-1 levels in 20 wk. old *Apoe*$^{-/-}$ mice fed normal chow. These levels (193.7±14.4 ng/ml, n=8) were not significantly different from those of 8 wk. old *Apoe*$^{-/-}$ mice indicating that the western diet raised circulating IGF-1. We used a commercially available assay (EMD Biosciences, San Diego, CA) to measure total cholesterol levels in serum from SMP8/*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice. SMC-specific IGF-1 overexpression did not change total cholesterol levels (*Apoe*$^{-/-}$, 1961±96 mg/dL, n=7; SMP8/*Apoe*$^{-/-}$, 1707±127 mg/dL, n=12; p=NS).

SMP8/*Apoe*$^{-/-}$ mice have increased vascular IGF-1 expression and aortic hypertrophy

After backcrossing SMP8-IGF-1 mice to the *Apoe*$^{-/-}$ background we confirmed persistent high-level expression of the IGF-1 transgene in SMP8/*Apoe*$^{-/-}$ mouse aorta by quantitative real-time RT-PCR (qPCR) with oligonucleotide pairs designed specifically for the rat

transgene. This transgene was detected only in SMP8/*Apoe*$^{-/-}$ mouse aorta and not in *Apoe*$^{-/-}$, whereas, levels of mouse endogenous IGF-1 transcripts, detected using murine-specific primers, were not different between SMP8/*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ (data not shown). We also performed qPCR using primers that were conserved between the two species and found that SMP8/*Apoe*$^{-/-}$ mice had an approximate 15-fold increase in IGF-1 mRNA levels compared with *Apoe*$^{-/-}$ (Fig 1D).

To determine the effect of SMC-specific IGF-1 overexpression on aortic morphology, we immunostained aortic arch cross-sections from SMP8/*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice for α-SMA, a SMC-specific cell marker. SMP8/*Apoe*$^{-/-}$ mice had 35% thicker aortic media ($P<0.01$), 51% larger aortic medial area ($P<0.01$), a 40% increase in lumen area ($P<0.05$), and a 23% increase in outer perimeter ($P<0.05$) compared to *Apoe*$^{-/-}$ mice (Table 1). We also assessed medial smooth muscle cell numbers and found that SMP8/*Apoe*$^{-/-}$ mice had a 35% higher number of medial smooth muscle cells per section (SMP8/*Apoe*$^{-/-}$; 762 ± 49 cells, n=5, *Apoe*$^{-/-}$; 565 ± 51 cells, n=5, $P<0.05$). Thus IGF-1 appears to proportionally increase the vascular size, consistent with the previous report on SMP8 mice.[22]

Targeted overexpression of IGF-1 in SMC does not alter atherosclerotic burden, plaque macrophage accumulation or oxidative stress in *Apoe*$^{-/-}$ mice

As shown in Fig. 2A, the percent of total plaque area in mouse aortas after 12 wks on a western diet was not different between SMP8/*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ animals. Likewise, morphometric analysis of aortic valve atherosclerotic plaques revealed that SMP8/*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice had similar lesion sizes (Fig. 2B). Analysis of aortic valve lesion size at 4 wks on a western diet also showed no difference between SMP8/*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice (Supplemental Figure 1). Since we have previously shown that infusion of IGF-1 in *Apoe*$^{-/-}$ mice reduced plaque macrophage accumulation, plaque TNF-α levels and aortic superoxide levels[21], we measured the plaque area staining positive for macrophages and found no difference between SMP8/*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice (Fig 2C). TNF-α-immunopositive plaque area was also not different between SMP8/*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice (Fig. 2C bottom panel).

To evaluate the potential effect of SMC-specific IGF-1 overexpression on oxidative stress in the atherosclerotic plaque, frozen aortic valve cross-sections from SMP8/*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice were stained with the superoxide-sensitive dye DHE with/without pretreatment with the superoxide-specific scavenger PEG-SOD. There were higher superoxide levels in the atherosclerotic plaque compared to adjacent media for both mouse strains (SMP8/*Apoe*$^{-/-}$, 40% increase, *Apoe*$^{-/-}$, 77% increase), but neither plaque nor medial superoxide levels were different in SMP8/*Apoe*$^{-/-}$ mice compared with those of *Apoe*$^{-/-}$ mice suggesting that increased SMC-specific IGF-1 expression did not alter vascular oxidative stress (data not shown).

Our previous findings indicated that IGF-1 infusion in *Apoe*$^{-/-}$ mice reduced aortic expression of the pro-inflammatory cytokines IL-6 and TNF-α, and markedly upregulated eNOS expression.[21] As shown in Table 1 SMP8/*Apoe*$^{-/-}$ mice had a similar reduction in aortic IL-6 and the same trend in TNF-α mRNA levels. However, unlike the effects of an increase in circulating IGF-1, SMC-specific IGF-1 overexpression failed to increase aortic eNOS expression.

SMC-specific IGF-1 overexpression increases features of plaque stability

To determine whether SMC-specific IGF-1 overexpression would alter lesion characteristics and namely accumulation of VSMC in atherosclerotic plaque we stained aortic valve cross-sections for VSMC marker proteins, αSMA, calponin, and SM22α. SMP8/*Apoe*$^{-/-}$ mice had a marked increase in αSMA-positive SMC area in atherosclerotic lesions (111 % increase

compared with *Apoe*$^{-/-}$ mice, p<0.001, Fig 3A) and a striking 120% increase in fibrous cap area (Fig. 3C). Areas of plaques staining positive for αSMA were also positive for calponin and SM22α, other markers of differentiated SMC (Fig. 3A). Calponin- and SM22α-positive plaque areas were increased by 164% and 93% in SMP8/*Apoe*$^{-/-}$ mice (Fig. 3A). The number of smooth muscle cells within plaques (defined as double positive for DAPI and αSMA) was increased in SMP8/*Apoe*$^{-/-}$ mice (SMP8/*Apoe*$^{-/-}$ mice; 99 ± 8, *Apoe*$^{-/-}$; 56 ± 10, n=9, p<0.01). This data suggests that the increase in smooth muscle marker expression is due to an increase in number of differentiated smooth muscle cells, although a contribution of smooth muscle cell hypertrophy can not be completely excluded. To further evaluate potential direct effects of IGF-1 on smooth muscle differentiation, cultured smooth muscle cells were exposed to 0–100 ng/mL IGF-1 and tested for smooth muscle marker expression. Intriguingly, IGF-1 enhanced smooth muscle myosin heavy chain, αSMA, calponin, and SM22α expression in a dose-dependent manner (Fig. 5), indicating that IGF-1 promotes SMC differentiation.

The increase in fibrous cap area was accompanied by a significant 36% reduction in necrotic core area/total plaque area (Fig. 3C) and by a marked 305% and 227% increase in plaque collagen content, as determined by trichrome staining and picrosirius red staining (imaged with polarized light), respectively (Fig. 3B). Imaging of picrosirius red stained sections using bright field microscopy showed a 406% increase (n=6, p<0.0001, not shown). Evaluation of plaque smooth muscle cell and collagen content at 4wks showed a similar increase in smooth muscle positive area (anti-αSMA staining, Supplemental Fig. 1B) and a strong trend to an increase in plaque collagen (Supplemental Figure 1F). Consistent with these in vivo observations, IGF-1 dose-dependently enhanced collagen type I and III expression in cultured aortic SMCs (Fig. 5B and 5C).

Since IGF-1 has strong anti-apoptotic effects in cultured aortic SMC[28] we measured SMC and total cell apoptosis in atherosclerotic plaques of SMP8/*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice. Total and SMC apoptotic rates were not different between the groups both at 12 wks (Fig. 4A) and at 4 wks time points (Supplemental Figure 1C and 1D). To obtain insights into potential mitogenic effects of IGF-1, we measured gene expression of the cell proliferation marker Ki67 in atherosclerotic aortas from SMP8/*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice and evaluated Ki67 levels in aortic valve plaques using immunohistochemistry with anti-Ki67 antibody. SMC-specific IGF-1 over-expression did not significantly change Ki67 expression in aortas (Table 1) and Ki67-positivity in atherosclerotic plaque (Ki67 expression in atherosclerotic plaque at 12 wks was barely detectable, data not shown). We also evaluated proliferating SMCs in plaques at 4wks using in vivo labeling with EdU, and did not find a significant difference (Supplemental Fig. 1F).

Discussion

The role of growth factors in atherosclerosis is pleiotropic. A permissive role is illustrated by their ability to stimulate SMC accumulation in developing lesions.[2–4, 29] This role is supported by results from mechanical injury models in which inhibition of growth factors such as PDGF[30–32] and IGF-1[11, 12] has resulted in blunted vascular neointimal responses and is the basis for strategies that have successfully been developed to treat restenosis after coronary interventions.[33, 34] However the role of growth factor stimulated plaque SMC accumulation in clinical events that are not related to restenosis remains unclear. In fact, clinical studies have indicated that plaques with a relative depletion of VSMC, thin fibrous caps and increased inflammatory cells are more prone to rupture.[35, 36] We have recently shown that infusion of IGF-1 reduced vascular oxidative stress, inflammation and atherosclerotic lesion size in *Apoe*$^{-/-}$ mice.[21] IGF-1, via its type 1 receptor, may act on many cell types involved in atherogenesis, including monocyte/macrophages, endothelial cells and VSMC. Its pleiotropic effects include stimulation of cell proliferation, differentiation, survival and metabolism.[9] Our

current finding that overexpression of IGF-1 in SMC in *Apoe*$^{-/-}$ mice on a western diet did not result in an increase in plaque burden is contrary to what could be expected based on the increase in IGF-1 expression seen after arterial balloon injury[6, 37] and the robust increase in neointima that occurs in the SMP8-IGF-1 transgenic mouse following mechanical injury.[16] Furthermore, while IGF-1 overexpression did not alter overall plaque burden it modified plaque composition by markedly increasing VSMC content.

The SMP8-IGF-1 mouse originally described by Wang et al[22] has a marked increase in IGF-1 expression in SMC of the gastro-intestinal tract, bladder, uterus and vasculature with a 3.5 to 4 fold increase in IGF-1 protein in the aorta and no change in plasma IGF-1 or IGF binding proteins.[22] This allowed us to specifically explore the effect of SMC autocrine/paracrine IGF-1 in atherosclerosis development/progression. After backcrossing this mouse to the *Apoe*$^{-/-}$ background, we confirmed persistent high level transgene expression. Morphometric analysis showed an increase in aortic outer perimeter, medial area, medial thickness, and lumen size, associated with a higher number of medial smooth muscle cells (Table 1). These findings are consistent with the growth effect of IGF-1 and are similar to those reported by Wang et al.[22] Of note, the increase in SMC mass in the SMP8-IGF-1 mouse was previously shown to be primarily due to SMC hyperplasia[22] and we confirmed an increase in medial SMC number. We found no change in circulating IGF-1 in SMP8/*Apoe*$^{-/-}$ mice compared with *Apoe*$^{-/-}$ mice. However, interestingly, circulating IGF-1 in *Apoe*$^{-/-}$ mice was increased after 12 wks on a Western diet. To our knowledge this finding has not previously been observed. Because circulating IGF-1 is primarily hepatic in origin, this finding suggests that the marked hypercholesterolemia and/or increase in oxidant stress present in *Apoe*$^{-/-}$ mice induces increased hepatic IGF-1 expression.

The majority of studies exploring mechanical injury models have demonstrated that an increase in IGF-1 or IGF-1 signaling correlates with increased neointimal burden.[14, 16] However, few studies have explored the role of IGF-1 in a hypercholesterolemic mouse model of atherosclerosis. Harrington et al[38] reported that a null mutation for the pregnancy-associated plasma protein-A gene, a metalloproteinase that degrades IGF binding protein 4, reduced lesion size in *Apoe*$^{-/-}$ mice fed a Western diet, potentially via a reduction in IGF-1 bioavailability. However, we have shown that infusion of IGF-1 to *Apoe*$^{-/-}$ mice reduced atherosclerotic plaque macrophage infiltration and lesion burden.[21] This atheroprotective effect was accompanied by a decrease in systemic and vascular oxidant stress, by reduced aortic expression of pro-inflammatory cytokines, and by an increase in aortic eNOS expression. Our present study indicates that these atheroprotective effects of IGF-1 are likely in large part mediated via non-SMC target cells, since overexpression of IGF-1 in SMC did not recapitulate most of these findings, with the exception of a reduction in aortic TNFα and IL-6 expression.

To our knowledge this is the first report of a growth factor being overexpressd in SMC in an atherosclerosis model and our results have significant implications for understanding the role of SMC accumulation in atherogenesis. Our findings suggest that this accumulation can be considered a repair mechanism and suggest that strategies to inhibit VSMC accumulation (outside of the specific condition of restenosis) may not be optimal. This may be of particular relevance to advanced atherosclerotic plaques, where a balance between cell death and survival of cells within the fibrous cap, primarily composed of VSMC and extracellular matrix, appears to correlate with plaque instability and stability.[2, 3, 29] Indeed, elegant studies from Clarke et al[39, 40] have shown that chronic apoptosis of VSMC accelerates atherosclerosis in *Apoe*$^{-/-}$ mice. Our findings indicate that IGF-1 overexpression in SMC increases SMC mass and collagen content and reduces necrotic cores in atherosclerotic plaque resulting in no change in overall plaque burden. Cellular apoptotic rates and indices of cell proliferation (Ki67 expression and EdU detection) were not different at 4 wks or 12 wks suggesting that increased proliferation or survival are not the primary determinants of increased plaque SMC content.

IGF-1 may induce VSMC migration[41] and VSMC in atherosclerotic plaque are less differentiated and stain poorly for marker contractile proteins, so it is possible that the marked increase in αSMA, calponin, and SM22α staining in plaques of SMP8/*Apoe*$^{-/-}$ mice represented IGF-1 induced increases in VSMC migration and/or differentiation. In fact, our in vitro study using cultured SMCs showed that IGF-1 dose-dependently enhanced smooth muscle marker protein expression, namely αSMA, calponin, SM22α and smooth muscle myosin heavy chain (Fig. 5). Our data are consistent with prior reports indicating that IGF-1 may regulate SMC phenotype in vitro[42–44] and are the first observations suggesting that IGF-1 promotes SMC differentiation in vivo. Our in vitro data also indicated that IGF-1 increased collagen type I and type III expression (Fig. 5B and 5C) and this was consistent with the marked increase in collagen in plaques from SMP8/*Apoe*$^{-/-}$ mice. Collagen production and SMC differentiation have been thought to be inversely related. In this regard, it is noteworthy that areas staining positive for SMC markers and collagen did not coincide in large part (Fig. 3). In fact, areas positive for SMC marker proteins localized mainly at the surface and shoulder of plaques, whereas core regions of plaques were heavily stained for collagen (trichrome and picrosirius red). Micro-environments for SMCs in a plaque are widely different according to their location inside the plaque; for instance, SMCs migrated into a core region of the plaque would be extensively exposed to extracellular materials, such as oxidized lipids, which potentially modify SMC phenotype [45]. It is thus possible that SMCs in different locations respond differently to IGF-1 as regards expression of marker proteins or synthesis of collagen. Intriguingly, cultured SMCs responded to IGF-1 by upregulating both SMC marker expression (Fig. 5A) and collagen type I and type III expression (Fig. 5B and 5C). This effect of IGF-1 appears to be analogous to that of lipid lowering. Indeed, Aikawa et al. have shown that lipid lowering in atherosclerotic rabbits produced an increase in SMC differentiation as evidenced by increased expression of smooth muscle myosin heavy chain (SM-MHC) and an increase in atherosclerotic plaque fibrillar collagen content[46, 47]. These effects were thought to be related to a reduction in both macrophage accumulation and expression of matrix metalloproteinases. It is also important to note that smooth muscle progenitors may be involved in extracellular matrix production[48, 49], and our study did not assess potential IGF-1 effects on these cells.

Our results clearly show that SMP8/*Apoe*$^{-/-}$ mice had no increase in atherosclerotic plaque burden and an unequivocal change in plaque composition, namely an increase in differentiated VSMC with an increase in collagen content and a reduction in necrotic core area. In fact, while there is significant debate about the usefulness of murine model systems for the investigation of acute events such as plaque rupture and thrombosis, these models, including the *Apoe*$^{-/-}$ mouse, are considered relevant tools to investigate processes of lesion progression. In particular, phenotypic characteristics of plaque, such as SMC content, fibrous cap thickness and collagen content are widely used as indirect indicators of plaque stability.[50, 51] It is of note that Tang et al[52] have shown that deletion of PDGF-B in circulating cells led to enhanced plaque inflammatory cell infiltration and delayed but did not ultimately inhibit SMC accumulation in plaque. Also, cardiac specific overexpression of TGF-β, a growth factor that stimulates VSMC proliferation and matrix protein production, limited atherosclerotic plaque burden in *Apoe*$^{-/-}$ mice.[53] Since aortic lesions in the mouse do not rupture, it will be important to evaluate brachiocephalic arteries in SMP8/*Apoe*$^{-/-}$ mice, where spontaneous plaque disruption has been reported [50, 51, 54], to demonstrate potential effects of IGF-1 on plaque stability.

Of course, translation of our findings to clinical studies to explore use of IGF-1 for plaque stability would require much further work. However, it is important to note that IGF-1 is approved for human use for growth disorders in children and the combination of IGF-1 complexed to its main circulating binding protein, IGFBP-3, may have advantages versus direct IGF-1 delivery. [55]

In summary, this is the first report of targeted overexpression of a growth factor to SMC in atherosclerosis models. Our data indicate that chronic overexpression of IGF-1 in SMC results in no change in atherosclerotic plaque burden in *Apoe*$^{-/-}$ mice fed a high-fat diet and induces a change in lesion composition with features consistent with more stable plaque. Our findings underscore differences in mechanisms between restenosis and atherosclerosis and suggest that stimulation of VSMC IGF-1 signaling may be beneficial for stabilization of atherosclerotic plaques. Our results also indicate that the reduction in atherosclerotic plaque burden produced by an increase in circulating IGF-1 is unlikely to be related to SMC specific effects of IGF-1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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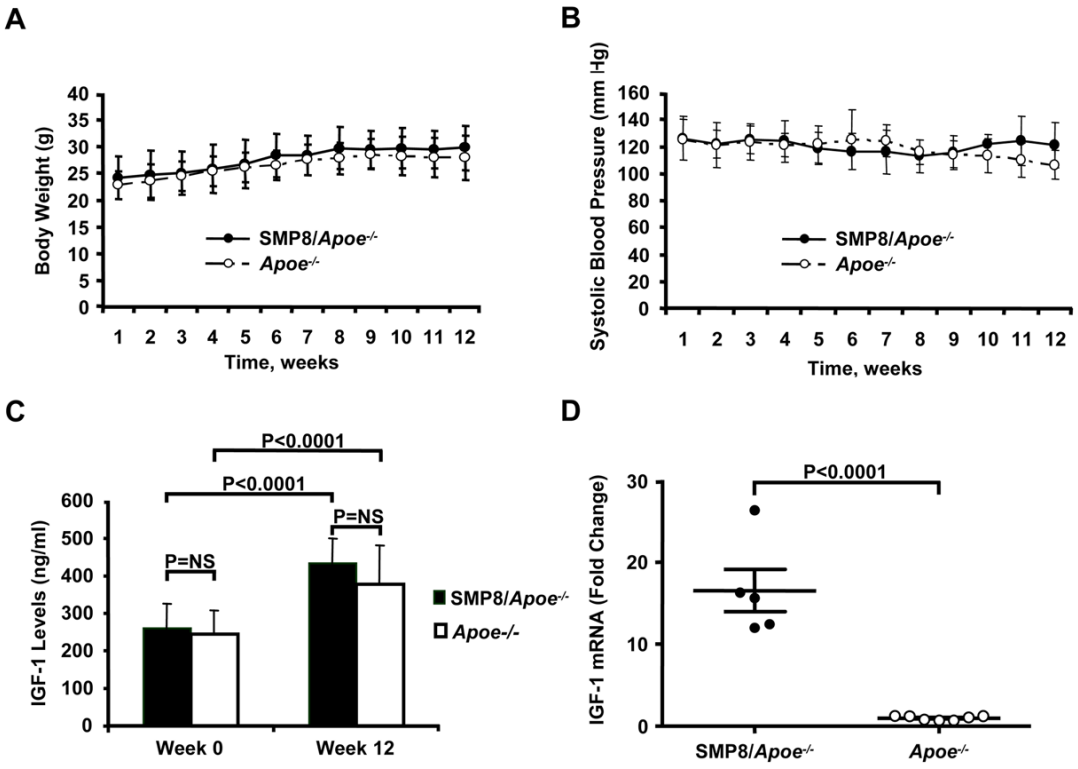

Figure 1. SMC-specific overexpression of IGF-1 does not change body weight, systolic blood pressure and circulating IGF-1 levels

8 wk old SMP8/*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice were fed with a Western diet for 12 wks. The body weight **(A)** and systolic blood pressure **(B)** were measured weekly and circulating IGF-1 **(C)** measured at weeks 0 and 12. **(D)** Change in aortic IGF-1 mRNA levels in SMP8/*Apoe*$^{-/-}$ mice compared with *Apoe*$^{-/-}$ mice, detected by qPCR using primers common for the rat and mouse IGF-1 gene.

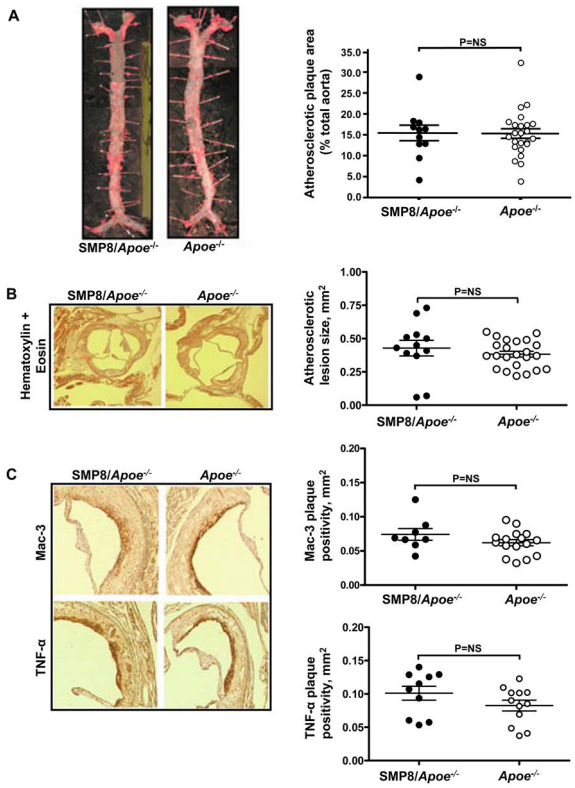

Figure 2. Overexpression of IGF-1 in SMC does not alter atherosclerotic burden, macrophage infiltration or TNF-α levels

(A) Representative Oil Red O stained *en face* aortae from SMP8/*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice (left) and quantitative assessment of plaque area (right). NS: not significant. Vertical bars are mean±SEM per group. **(B)** Representative aortic root cross sections from SMP8/*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice (left, magnification X10) and quantitative assessment of plaque area (right). **(C)** Representative staining of aortic root cross sections (magnification ×20) for macrophages (anti-Mac-3 antibody, top panels, left) and TNF-α (bottom panels, left) and quantitative assessment of Mac-3-positive lesion area (top graph) and TNF-α positive lesion area (bottom graph).

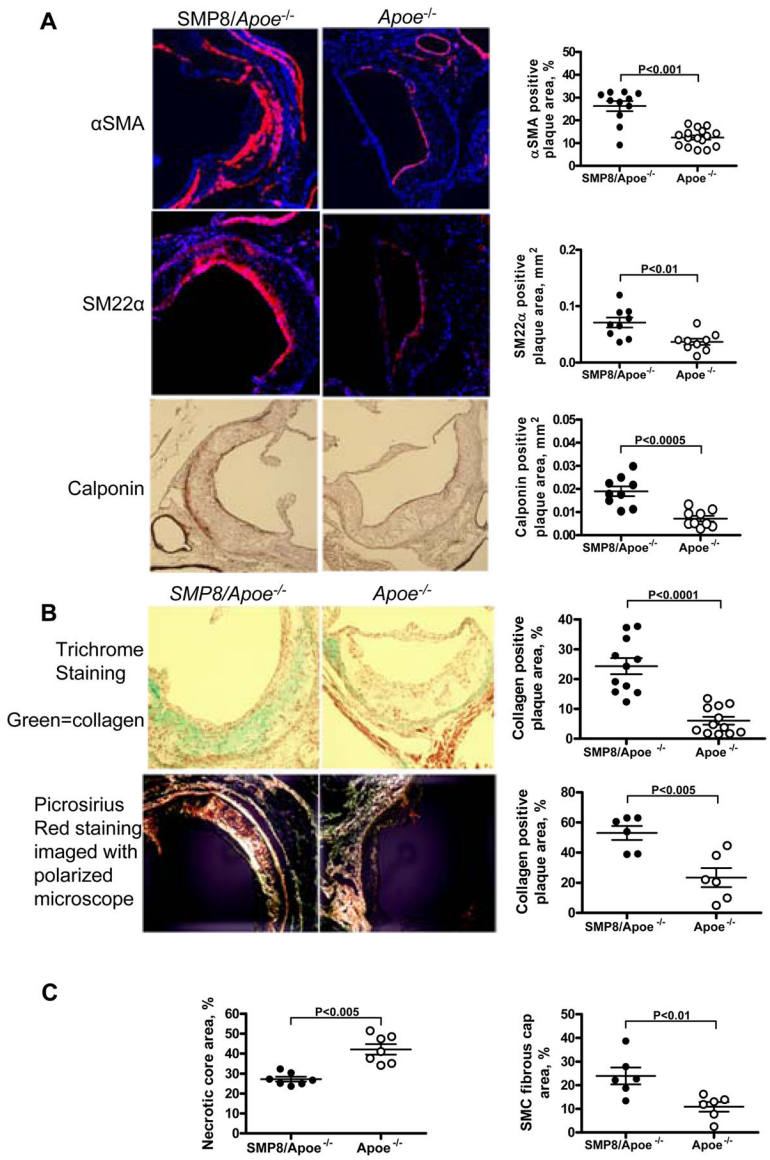

Figure 3. IGF-1 overexpression increases SMC and collagen content and fibrous cap area and reduces necrotic cores in atherosclerotic plaque

(A) Representative staining of aortic valve cross sections for αSMA (top panels, left), SM22α (middle panels, left), and calponin (bottom panels, left), and graphs (right) show quantitative data indicating αSMA, SM22α, and calponin positive plaque areas, respectively. Vertical bars are mean±SEM per group.

(B) Trichrome staining (top panels, left) and picrosirius red staining (bottom panels, left) for collagen (Magnification ×20). Graphs (right) show quantitative data indicating trichrome (SMP8/*Apoe*$^{-/-}$; n=11, *Apoe*$^{-/-}$; n=12) and picrosirius red positive areas as percentage of plaque area. Vertical bars are mean±SEM per group.

(C) Quantitative data show necrotic core area (left) and SMC fibrous cap area (right) as percentage of atherosclerotic plaque area. Vertical bars are mean±SEM per group.

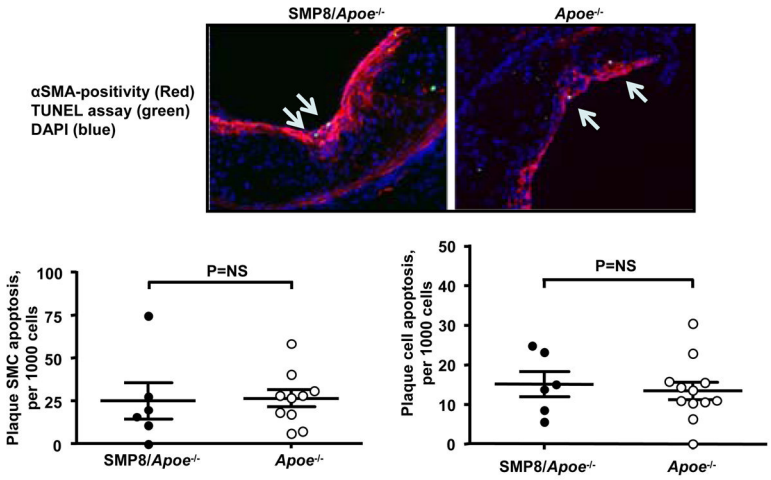

Figure 4. IGF-1 overexpression does not alter total and SMC apoptosis in atherosclerotic plaque

Representative aortic valve cross sections stained with TUNEL, anti-αSMA antibody and DAPI (top panels, arrows indicate apoptotic SMC). Graphs indicate total cell apoptosis defined as TUNEL-positive cell number per 1000 plaque cells (bottom, right) and SMC apoptosis measured as the number of αSMA/TUNEL-double positive cells per 1000 αSMA-positive cells (bottom, left). Vertical bars are mean±SEM per group.

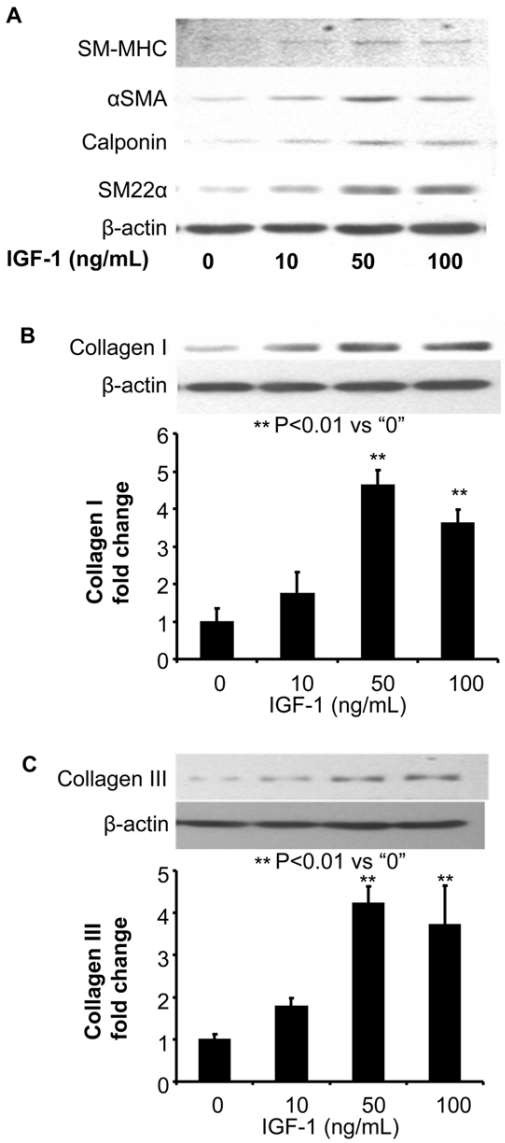

Figure 5. IGF-1 enhances SMC contractile protein expression and collagen type I and III expression in cultured SMCs

(A) IGF-1 dose-dependently upregulated smooth muscle myosin heavy chain (SM-MHC), αSMA, calponin, and SM22α expression in cultured vascular SMCs. Shown are representative data from 3 independent experiments. IGF-1 dose-dependently enhanced collagen type I **(B)** and collagen type III **(C)** expression in cultured vascular SMCs. Densitometric analysis was performed (graphs) and results are indicated as mean±SEM.

Table 1

Morphometry and Gene Expression Changes in Aortae from SMP8/Apoe$^{-/-}$ and Apoe$^{-/-}$ Mice

Morphometry	**SMP8/Apoe$^{-/-}$**	**Apoe$^{-/-}$**	**P value**
Medial area, mm^2	0.343 ± 0.052	0.227 ± 0.026	0.008
Lumen area, mm^2	0.728 ± 0.089	0.520 ± 0.042	0.045
Medial thickness, μm	93.88 ± 0.35	69.50 ± 3.88	0.005
Outer perimeter, mm	1.569 ± 0.127	1.280 ±0.052	0.041
mRNA Expression	***SMP8/Apoe$^{-/-}$***	***Apoe$^{-/-}$***	***P value***
eNOS	1.049 ± 0.16	1.000 ± 0.27	0.88
IL-6	0.2945 ± 0.05	1.000 ± 0.21	0.04
TNF-α	0.5833 ± 0.07	1.000 ± 0.21	0.20
Ki67	0.9085 ± 0.26	1.000 ± 0.18	0.77