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Collagen biosynthesis by neointimal smooth muscle cells cultured from rabbit aortic explants 15 weeks after de-endothelialization

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Summary. Extracellular matrix (ECM) accumulation in arterial neointima, developed in response to de-endothelialization, is a prolonged process. In this study, we examined the relationship between increased collagen accumulation and synthetic activity of neointimal smooth muscle cells (SMCs) derived from aortic explants fifteen weeks after balloon catheter injury. Freshly confluent SMCs, derived either from normal aorta or from aortic neointima, were used in this study. The newly synthesized collagen was analysed by measuring [^{3}H]-proline incorporation; and the mRNA expression for two major types of collagen, collagen type I and type III, was studied by Northern blot analysis. Our results indicated a three fold increase in protein (collagen) synthesis by neointimal SMCs. At the same time, the steady-state mRNA for procollagen I and procollagen III was elevated five and three times, respectively. These data indicate that persistent synthesis contributes to collagen accumulation in the arterial neointima and both transcriptional and post-transcriptional regulation take part in this process.

Keywords: collagen, ECM, neointima, mRNA, atherosclerosis

Atherosclerosis is characterized by focal thickening of musculo-elastic arteries accompanied by accumulation of extracellular matrix (ECM) components and lipoproteins (Katsuda *et al.* 1992; Alavi *et al.* 1992). Collagen constitutes the major ECM protein both in normal artery and in atherosclerotic lesions. A number of studies suggest that collagen plays an important part in atherosclerosis, not only because of its structural stabilization function in plaques, but also because of its interaction with growth factors, cytokines and lipoproteins (Barnes 1985; Moore & Alavi 1995). As a family, collagen comprises at least 15 distinct members. Six of them are present in the vascular wall, namely types I, III, IV, V, VI and VIII collagen (Tan & Uitto 1989). Both SMCs and endothelial cells synthesize collagen, but SMCs produce the major amounts of arterial collagen (Desmouliere & Gabbiani 1992; Mayne 1986).

The metabolism and distribution of various types of collagen in atherosclerotic lesions has been extensively studied. Elevated collagen concentration has been shown in atherosclerotic lesions (Katsuda *et al.* 1992; McCullagh *et al.* 1980). Among all the types of collagen, increased deposition of collagen type I and III is most predominant. Most reports indicate that these two types of collagen represent 60% of the total protein and 80–90% of the total collagenous protein in atherosclerotic

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lesions (Rauterberg & Jaeger, 1992). Further observations indicated a decreased proportion of type III to type I collagen in lesions (Stiemer *et al*. 1993). Most of these results were obtained from biochemical analysis using aortas from autopsy. An inherent disadvantage relates to the presence of other components from arterial intima and adventitia, which can only be overcome by using pure cultures of SMCs.

Quantitation of various types of collagen usually employs pepsin solubilization, followed by electrophoresis or chromatography. Conflicting data are generated, which reflect the technical limitations of the methodology used (Tan & Uitto 1989). Recent developments in molecular biology provide us with a powerful tool to analyze collagen synthesis. All the known types of collagen can be easily differentiated at the nucleic acid level and their amount can subsequently be measured (Linsenmayer 1993).

Using a nucleic acid hybridization technique, we examine the biosynthesis of collagen type I and type III by cultured SMCs from the neointima, fifteen weeks after a selective arterial de-endothelialization of the rabbit aorta by a balloon catheter. Our results suggest that de-endothelialization of aortic intima stimulates collagen synthesis and this induced stimulation persists for 15 weeks after a single injury. Further, the effects of the de-endothelialization appear more pronounced at transcriptional level than the effects at translational level.

Methods

Animals and surgical procedure

New Zealand, white, male rabbits, 2.0–2.5 kg body weight, were acclimatized to the animal quarters for at least two weeks. The surgical procedure to remove endothelium, identical to that reported previously (Alavi *et al*. 1992; Li *et al*. 1995), was performed on a group of ten rabbits. Briefly, the animals were anaesthetized with pentobarbital sodium (30 mg/kg b.w.). One femoral artery was exposed and a small incision was made. Then a 4F Fogarty, arterial, embolectomy catheter (American Edwards Laboratories, Santa Ana, Ca, USA) was introduced through the incision up to the arch of the aorta. The balloon catheter was inflated with 0.75 ml saline and withdrawn with decreasing volume. The procedure was repeated three times to ensure complete de-endothelialization. The catheter was then removed and the incision was closed. Another group of ten rabbits, used as controls, was subjected to a sham operation, i.e. the femoral artery was exposed but no catheter was inserted. Animals of both groups were maintained on normal rabbit chow and water *ad libitum* for the rest of the experimental period.

All animal procedures carried institutional approval according to guidelines set by the Canadian Council on Animal Care.

Tissue preparation and smooth muscle culture

The rabbits from both groups i.e. sham operated or de-endothelialized were killed with an overdose of pentobarbitol (30 mg/kg body weight) at 15th week after initial surgery. Thirty minutes before killing, the animals were injected with 5 ml of a 0.45% solution (wt/vol) of Evans blue dye (Allied Chemical Company, New York), which outlines with precision the areas where endothelium has not regenerated (de-endothelialized aorta, DEA) by staining them blue, whereas the areas where endothelium has regenerated (re-endothelialized aorta, REA) appeared white on a blue background. Five rabbits from each of the two groups were used for analysis of protein synthesis and Northern blot analysis was done on the remaining five rabbits from each group. After killing, the aortas from arch to bifurcation were removed *en bloc* and were then opened longitudinally. Tissues from DEA and REA were separated; those for morphological observation were rinsed, embedded in OCT and stored at -70°C; the tissues for culture were rinsed and immediately processed.

The SMC culture was performed as described previously (Li *et al*., 1993). Briefly, endothelium was removed from all arterial tissues, and the upper medial layer of control aorta and the neointimal layer of the balloon-injured aortas were peeled off using Bergh cilia forceps. The tissues from control arteries, from DEA or from REA, were separately cultured. The tissue explants were first cut into 1.0 mm^3 pieces and the pieces were then plated in 100 mm^2 culture dishes in DMEM containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B. The dishes were incubated at 37°C, in 5% CO_2 and 95% air. The medium was changed twice a week. When the cells reached relative confluence in primary culture, they were harvested with 0.25% trypsin-EDTA (GIBO, Burlington, Ont., Canada) treatment and an equal number of 1×10^5 cells was re-seeded into 35 mm Petri dishes. The cells from 1–3 passages were used for further analysis.

Immunofluorescence

A certain number of first passage SMCs were allowed to grow on coverslips in 35-mm Petri dishes at a density of

1×10^5 cells per dish. After reaching confluency, the cells were rinsed with PBS and fixed in 100% methanol for 5 min at room temperature, then air dried for at least 1 hr. After several rinsings with PBS, the cells were incubated for 90 min with a monoclonal, anti-α-smooth muscle actin antibody (Sigma, St Louis) at a concentration of 1:400 in PBS. As control, the cells in several dishes were incubated with non-immune rabbit serum. Fluorescent conjugated anti-mouse IgG antibody (FITC, Sigma, St Louis) was applied at a concentration of 1:32 in PBS. After 30 min incubation with secondary antibody, the coverslips, on which the cells grew, were rinsed several times and mounted on glass slides with 90% glycerol. The slides were observed on a fluorescence microscope (Leitz, Germany).

Collagen synthesis-protein analysis

We analysed collagen synthesis with a slight modification of the method described by Okada *et al.* (1990). The primarily cultured SMC cells were harvested and re-seeded as described above. All the measurements were started when the cells, from normal artery, DEA, and REA, had just reached a confluent state. Twenty-four hours before analysis, the cells were washed three times with fresh DMEM, and incubated with 1.0 ml DMEM containing 10% FBS, 20 μCi/ml of [H^3]-proline, supplemented with 0.1 mM sodium ascorbate and 0.5 mM β-aminopropionitrile fumarate. Then the cells were detached with trypsin-EDTA and the cell numbers were counted. The ice-cold cell suspension was homogenized by a sonicator (Quigley-Rochester Inc. Rochester, NY). The homogenized sample was used for quantitative measurement of collagen content.

Radioactivity counting

One aliquot of sample was heated at 100°C for 10 min and dialyzed thoroughly against 0.05 M acetic acid for 3 days at 4°C with gentle stirring. The samples were then collected and lyophilized in a Freeze Dryer (Lab Con Co., Canada). The lyophilized samples were redissolved in 1.0 ml 0.04 N NaOH, neutralized with Tris-HCl buffer (pH 7.4) and digested with collagenase (type VII collagenase, Sigma, St Louis, MO) at 37°C for 2 h. An aliquot of a 100 μl sample from collagenous fractions was mixed with 5.0 ml of cytoscint scintillation fluid (ICN Biomedicals, Costa Mesa, CA). The radioactivity was counted on a 1211 RACKBETA Scintillation counter (LKB, Sweden). Each sample was assayed in triplicate. The results were expressed as dpm/10^5 cells.

RNA extraction and Northern blot analysis

Cytoplasmic RNA was extracted using TRIzol Reagent (GIBCO BRL) according to Chomcynski's method (Chomcynski & Sacchi, 1987). RNA thus obtained was further purified by digesting with DNase (Progema, WI) for 60 min at 37°C. The purified RNA was separated on a 1% agarose gel containing 0.6 M formaldehyde and then transferred onto a nylon membrane (Hybond-N+, Amersham) using a semi-dry 2117 MULTIPHOR II electrophoresis unit (LKB). The membrane, with RNA immobilized by UV cross-linking, was hybridized to the respective cDNA probe. The plasmids containing cDNA of human collagen type I(α1) and type III(α1) clones were purchased from ATCC (American Type Culture Collection). The subcloning and cDNA probe preparations were performed according to the depositors' instruction. Radiolabelling of the probes was done using [α-^{32}P]-dCTP (ICN) and T7 random labelling kit (Pharmacia). Autoradiography was performed by exposing X-ray films (Kodak X-Omat AR), with intensifying screens, at -70°C. The exposure time was 1 week. Autoradiographs were scanned using a Hoefer GS 3000 densitometer (LKB) to measure the mRNA signals with α-tubulin hybridization as a reference for the relative mRNA load per lane.

Statistical analysis

All data were expressed as mean $\pm$ SD. Statistical significance was determined with one way ANOVA and p values less than or equal to 0.05 were considered significant.

Results

Morphological observation

The intimal layer in normal rabbit aortas is very thin, consisting of a single layer of endothelium. After arterial injury, however, neointimal tissue was developed on the luminal side of the aorta having 3–4 layers of cells. Most of these cells appear elongated and are spindle-shaped, exhibiting the typical SMC phenotype.

In culture, growth of SMCs from aortic explants became visible at about 7 days in primary culture. The cells were elongated and grew radially from the explants. Figure 1 illustrates the immunofluorescence labelling of cultured cells with anti-α-smooth muscle actin antibody. The uniform and intense fluorescence reactivity confirms the SMCs nature of these cells.

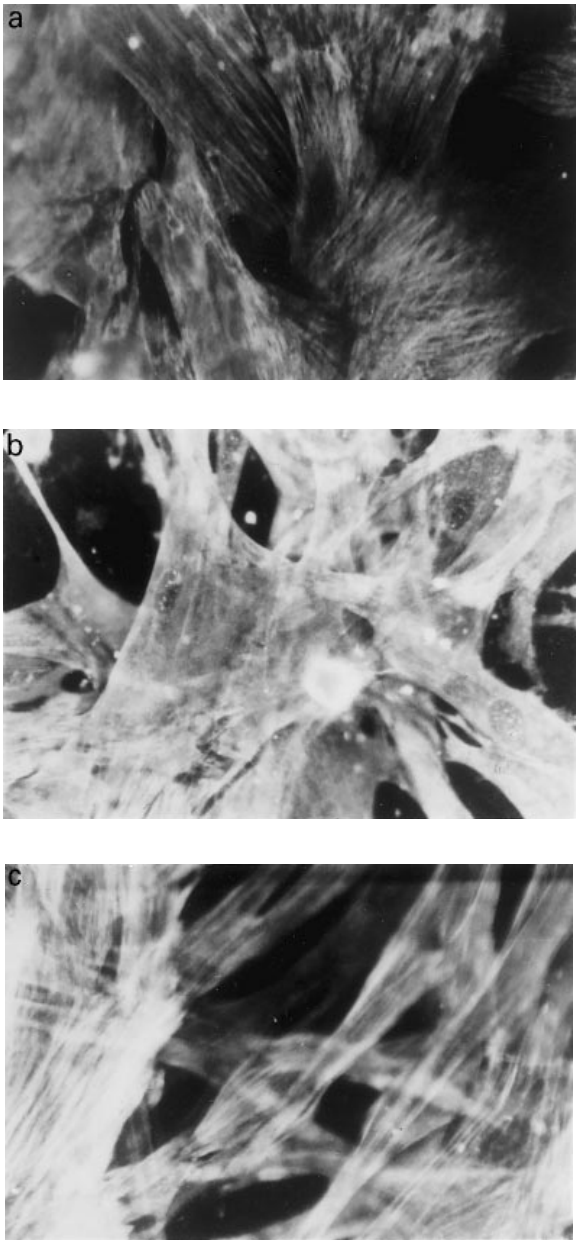

Figure 1. Immunofluorescent micrographs of cultured SMCs. All cells show reaction with a specific anti-α-smooth muscle actin antibody. a, normal SMCs; b, SMCs from DEA; c, SMCs from REA. The SMCs from DEA and REA tend to grow in an overlapping pattern (original magnification, $\times$400).

Collagen synthesis

The newly synthesized collagen, labelled with [^{3}H]-proline, was measured in SMC cultures from both normal and injured arterial tissues. All three types of SMCs synthesized measurable amounts of collagen. However, the neointimal SMCs (both from REA and DEA) synthesized more than twice the amount of collagen than did the control SMC ($P < 0.01$). The difference between REA and DEA was not significant. Results of the measurement are shown graphically in Figure 2.

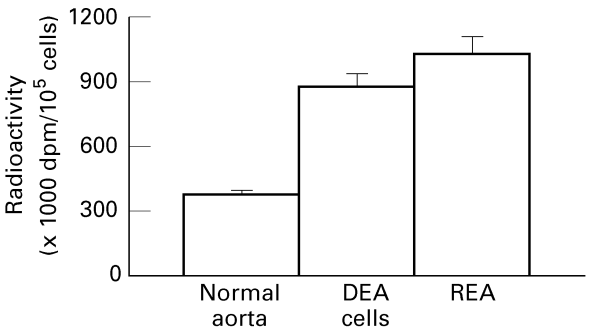

Figure 2. [^{3}H]-proline labelled collagen synthesised by SMC in culture and expressed as dpm/10^5 cells. The bars represent mean and standard deviation of incorporated radioactivity ($n = 5$).

Collagen gene expression

Signals for rabbit pro-α1(I) mRNA, transcripts of 4.8 kb and 5.8 kb, were present in SMCs from both normal and injured aortas. However, the intensities of the signals were different. The SMCs from REA had the highest intensity, followed by cells from DEA. The signals in SMCs from normal aortas were the weakest among the three, but they were still clearly visible in the autograph (Figure 3a). The intensities of signals from the different tissues were measured using a densitometer and normalized to the signals for mRNA of α-tubulin and 28S rRNA. As shown in Figure 3b, the signal intensities in SMCs from DEA and REA were 5 and 6 times stronger than those from normal aorta, respectively.

Signals for pro-α1(III) mRNA were transcripts of 5.4 kb and 4.8 kb. Even though this mRNA was expressed in all 3 types of SMCs, the signals in normal SMCs were weak and became visible by autograph only after 2 weeks of exposure (Figure 4a). Quantitative assessment showed that mRNA expression in neointimal SMCs was 3 fold higher (Figure 4b, $P < 0.05$) than in the normal SMCs. The difference between REA and DEA, however, was not significant.

Discussion

In this study, we noticed that neointimal SMC synthesized twice the amount of collagen than cells derived from normal artery fifteen weeks after de-endothelialization.

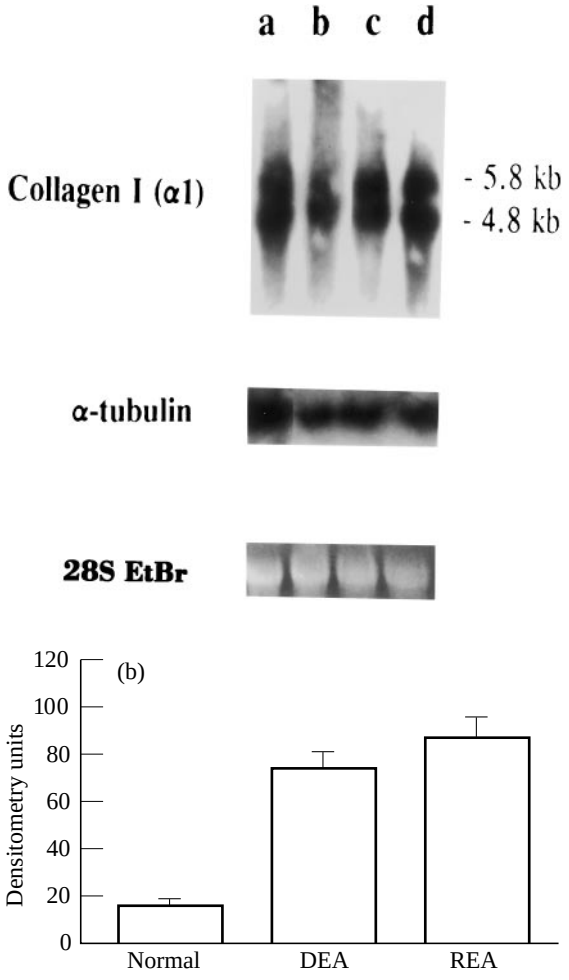

Figure 3. Northern blot analysis of Collagen type I mRNA expression in normal and neointimal SMCs. a, Cytoplasmic RNA from human fibroblasts was used as a positive control (lane a). RNA from normal SMCs, SMCs from DEA and from REA was loaded onto lanes b, c, d respectively. The blot was densitometrically scanned and normalized to α-tubulin and 28S rRNA for quantification. b, Histogram showing signals for Collagen type I is significantly increased in neointimal SMCs ($P < 0.01$). Values are represented as mean $\pm$ SD (normal SMC, 14.9 $\pm$ 1.2; SMCs from DEA, 76.0 $\pm$ 6.5; SMCs from REA, 89 $\pm$ 9.1; $n = 5$).

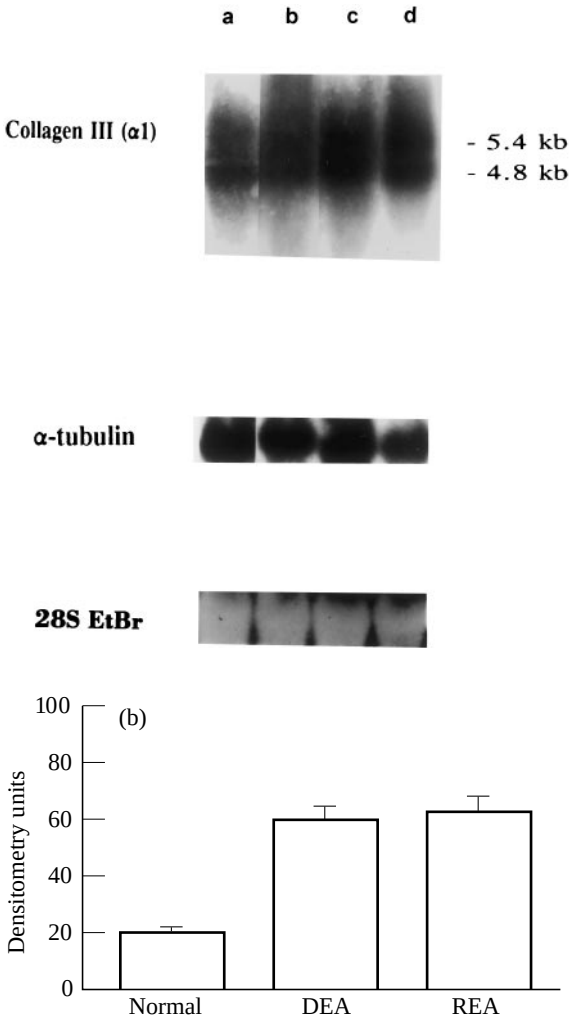

Figure 4. Northern blot analysis of Collagen type III mRNA expression in normal and neointimal SMCs. a, Cytoplasmic RNA from human fibroblasts was used as a positive control (lane a). RNA from normal SMCs, SMCs from DEA and from REA was loaded to lanes b, c, d respectively. The blot was densitometrically scanned and normalized to α-tubulin and 28S rRNA for quantification. b, Histogram showing signals for Collagen type I is significantly increased in neointimal SMCs ($P < 0.01$). Values are represented as mean $\pm$ SD (normal SMC, 20.9 $\pm$ 2.2; SMCs from DEA, 57.7 $\pm$ 4.3; SMCs from REA, 61.4 $\pm$ 5.1; $n = 5$).

In addition, the de-endothelialization altered the gene expression ratio of collagen type I and type III. De-endothelialization induced a 5–6 fold increase in pro α1 (I) mRNA and about a 3 fold increase in pro α1 (III) mRNA. These data indicate that the collagen type I to type III ratio is higher in the neointima, resembling early atherosclerotic plaque formation in man.

Balloon catheter de-endothelialization is widely employed for the induction of atherosclerosis in animals. This injury has been reported to initiate a complex cascade of interactions and events resembling those seen in atherosclerosis (Moore 1981; Moore & Alavi 1995). In rabbits, we and others have shown that this manipulation leads to SMC migration, proliferation, ECM accumulation and thus neointimal formation. In long term experiments, we have also shown deposition of lipoprotein in the neointimal tissue of normally fed rabbits (Richardson *et al.* 1989). Numerous reports have documented the similarities between neointimal and

atherosclerotic SMCs. For example, both cell types are rich in endoplasmic reticulum, Golgi apparatuses and ribosomes; at the same time, both of them have decreased myofilament volume compared with their cells of origin. Probably more importantly, both cell types have enhanced synthetic ability and produce more ECM components (Campbell & Campbell, 1985). A major concern about neointimal SMCs is the duration of their phenotypic change. Results from this laboratory indicate that SMC phenotypic change might be a persistent process. For example, a recent study from this laboratory has shown that neointimal SMCs, 15 weeks after initial de-endothelialization, retained an enhanced ability to synthesize proteoglycan (Li *et al*. 1994). Our results of enhanced collagen synthesis 15 weeks after endothelial injury further support the persistence of the phenotypic alteration of SMCs.

Many studies of collagen production in aortic tissues or cells have been performed by measuring the metabolic incorporation of radiolabelled proline into hydroxyproline. The latter is employed as an index of collagen synthesis (Okada *et al*. 1990). This method is effective in measuring newly synthesized collagen by various tissue and organ cultures, but it is not able to identify the change of any particular type of collagen. Two approaches are commonly applied in characterization of various types of arterial collagen. The first involves immunohistochemical staining using type-specific anti-collagen antibodies (Stiemer *et al*. 1993). Obviously, this method does not allow quantitative evaluation of collagen. The second approach utilizes pepsin solubilization of ECM followed by sodium dodecyl sulphate (SDS) gel electrophoresis, or chromatography. However, the extent of pepsin proteolysis is hard to control and data derived, employing this method, are conflicting. In contrast to the complex biochemical analysis, all types of collagens can be easily separated at nucleotide level. In fact, certain types of collagen were first identified at the molecular level (Yoshioka & Ramirez 1990). So, the nucleotide hybridization technique, such as Northern blot analysis, provides a powerful quantitative tool for the analysis of various types of collagen (Rekhter *et al*. 1993).

The increased collagen synthesis observed in this study may relate to altered production of growth factors and cytokines and their receptors. These mitogens can up-regulate collagen production at both transcriptional and post-transcriptional level (Chang & Sonenshein 1991). Transforming growth factor-β1 and platelet derived growth factor (PDGF) are two important regulators implicated in the development of atherosclerosis. Schlumberger *et al*. (1991) reported the enhancement of production of collagen and noncollagen proteins by the co-culture of arterial SMCs and TGF-β1; moreover the increased rate of net collagen production was twice as high as that of noncollagenous proteins. PDGF is composed of two related polypeptides, designated as A and B chains. Amento *et al*. (1991) showed that PDGF could stimulate collagen synthesis in cultured SMCs. The stimulatory effect of PDGF is largely mediated by its proliferation-promoting function. Employing a solid-phase ELISA method, we have recently shown that de-endothelialization stimulates the SMC production of TGF-β1 and PDGF-AB fifteen weeks after de-endothelialization (Li *et al*. 1995). In another study, we also showed that de-endothelialization upregulates the mRNA expression of the PDGF-β receptor about 2 fold (Alavi *et al*. 1994). Besides SMCs, macrophages and lymphocytes in the atherosclerotic plaque can produce various growth factors and cytokines (Hansson *et al*. 1991; Ross *et al*. 1990). Persistent expression of growth factors and their receptors is likely responsible for increased collagen synthesis.

As the major ECM protein in atherosclerotic plaque, collagen contributes significantly to the occlusive nature of atherosclerosis. Besides, collagen facilitates lipoprotein retention in the lesions. It has been shown that type I and type III collagen have a high affinity for LDL, especially for oxidized LDL (Jimi *et al*. 1994). Co-localization of fibrillar collagen and lipid-vesicles in both early and late atherosclerosis has been observed (Guyton & Klemp 1993). In our model, de-endothelialization also causes direct contact of collagen with platelets. This contact activates platelets and causes the secretion of factors like PDGF, TGF-α, TGF-β and TxA_2, which stimulate SMC proliferation, migration and thus accelerate atherogenesis (Raines & Ross, 1993).

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