

Transforming growth factor- β 1 gene and protein expression associated with atherogenesis of cholesterol-fed rabbits

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Summary. Transforming growth factor- β 1 (TGF- β 1) has been shown to modulate both cell proliferation and the synthesis of extracellular matrix by vascular cells. This study was aimed to establish the temporal correlation between TGF- β 1 expression, the expression of the extracellular matrix protein fibronectin, and plaque development during atherogenesis of hypercholesterolemic rabbits. New Zealand White rabbits were fed with 2% cholesterol-supplemented chow for 1 week, 2 weeks, 3 weeks or 6 weeks. TGF- β 1 mRNA and protein expression was examined in serial sections of aorta by in situ hybridization and immunohistochemistry. Fibronectin expression was examined by immunohistochemistry. In the control and 1-week feeding group, the expression of TGF- β 1 mRNA and protein was not apparent. In 2-week feeding group, intimal thickening was detected in which TGF- β 1 mRNA and protein were not clearly observed, either. The 3-week and 6-week feeding groups exhibited fatty streaks in which TGF- β 1 mRNA and protein expression markedly increased as feeding proceeded. Cell type-specific staining indicated that TGF- β 1 was expressed by macrophages as well as smooth muscle cells of the fatty streaks. Immunostaining of fibronectin detected low expression levels in control, 1-week and 2-week feeding groups with pronounced upregulation in the thickened intima and the proximal media in 3-week and 6-week feeding groups. These results implicate a role for TGF- β 1 in modulating fatty streak formation and the synthesis of extracellular protein fibronectin during plaque development.

Key words: Transforming growth factor- β 1, Atherogenesis, Fibronectin, In situ hybridization, Immunohistochemistry

Introduction

Atherosclerosis is a pathophysiological process of the vasculature underlying a variety of diseases including myocardial and cerebral infarctions. The development of atherosclerotic lesions has been shown to involve endothelial injury, the recruitment of blood monocytes and lymphocytes, the migration and proliferation of smooth muscle cells and the interplay among these cells through the release of cytokines and growth factors (Ross, 1993). The earliest recognizable atherosclerotic lesions are fatty streaks whose locations are thought to be highly correlated with those of focal intimal thickening of the arterial wall (Stary et al., 1992). Fatty streaks are characterized by the accumulation of lipid droplets in foam cells derived from macrophages and smooth muscle cells in both human and animal models (Faggiotto et al., 1984; Aqel et al., 1985; Aliev and Burnstock, 1998). The activation of macrophages and smooth muscle cells plays a pivotal role through the development of atherosclerosis.

Transforming growth factor- β 1 (TGF- β 1) is a multifunctional cytokine which has been demonstrated to modulate the proliferation of a variety of cell types and to regulate their interaction with the extracellular matrix (Sporn et al., 1986). Experimental evidence indicated that TGF- β 1 is a potent stimulator for the synthesis of proteoglycans (Chen et al., 1987; Schonherr et al., 1991) and various extracellular matrix proteins (Balza et al., 1988; Lawrence et al., 1994) as well as a modulator for proteases associated with extracellular matrix proteins (Edwards et al., 1987). In addition to its prominent role in regulating extracellular matrix formation, TGF- β 1 has been previously reported to either promote or inhibit growth, depending on cell age, cell density, the presence of coexisting factors, and the concentration of TGF- β 1 (Mii et al., 1993; Halloran et al., 1995). TGF- β 1 was found to decrease proliferation rate of cultured arterial smooth muscle cells by extending the G2 phase of cell cycle (Grainger et al., 1994). On the other hand, expression of active TGF- β 1

in normal arteries following gene transfer was found to markedly stimulate extracellular matrix synthesis and, to a lesser extent, cellular hyperplasia of the intima and media (Nabel et al., 1993). An enhanced intimal thickening was also observed in arteries injured with balloon catheter following the administration of TGF- β 1 (Kanzaki et al., 1995). Another prominent function of TGF- β 1 is its strong chemoattractive effects on circulating monocytes (Wahl et al., 1987; Wiseman et al., 1988). In this context, the disruption of the gene encoding TGF- β 1 in mouse resulted in a systemic inflammatory disease (Shull et al., 1992).

The role of TGF- β 1 in arterial restenosis following therapeutic interventions has been strongly implicated. In animal models, vascular injury caused by balloon angioplasty induced a pronounced upregulation of TGF- β 1 by arterial smooth muscle cells (Majesky et al., 1991; Wolf et al., 1994; Kanzaki et al., 1995), and anti-TGF- β 1 antibodies were found effective in suppressing intimal hyperplasia (Wolf et al., 1994). Furthermore, stimulation of TGF- β 1 was shown to be especially prominent in the restenosis lesions of human coronary arteries (Nikol et al., 1992). In contrast to its well-established role in arterial restenosis, the involvement of TGF- β 1 in the primary atherosclerosis is not as clear. Using the reverse transcription-polymerase chain reaction analysis, it was reported that both TGF- β 1 and TGF- β 2 mRNA expression was upregulated in the aortic arches of Watanabe rabbits fed with 0.5% cholesterol-supplemented chow for two months (Lopez-Candales et al., 1995). The time course and cellular source of TGF- β 1 during the initial development of atherosclerotic lesions, however, remain unknown. Therefore, we examined TGF- β 1 mRNA and protein expression in hypercholesterolemic rabbits following the feeding of a 2% cholesterol-supplemented diet for 1 week, 2 weeks, 3 weeks or 6 weeks. The expression of fibronectin during atherogenesis was also examined for comparison. Our results demonstrated that vascular TGF- β 1 mRNA and protein expression is upregulated in the fatty streaks whereas a pronounced upregulation for fibronectin was detected in both proximal media and the thickened intima. These results are consistent with TGF- β 1 playing a role in modulating the development of atherosclerotic lesions.

Materials and methods

Experimental animals

Hypercholesterolemia of forty male New Zealand White rabbits (2 kg body weight) was induced in the experimental groups by feeding a regular diet supplemented with 2% (wt/wt) cholesterol (Purina Mills, USA). Each group of ten rabbits was fed with the diet for 1 week, 2 weeks, 3 weeks, and 6 weeks, respectively. Twenty age-matched rabbits fed on regular rabbit chow were used as control.

Following the treatment, rabbits were anesthetized

with the intravenous injection of 35-40 mg/kg sodium pentobarbital. Aortic arch was removed, dissected gently free of adhering tissues, and rinsed with ice-cold phosphate buffered saline (PBS). Each aortic arch was immersion-fixed with 4% buffered paraformaldehyde and paraffin-embedded for in situ hybridization and immunohistochemistry. The investigation conforms with the "Guide for the care and use of laboratory animals" published by the US National Institute of Health.

In situ hybridization analysis and immunohistochemistry analysis

To examine the cellular expression and localization of the TGF- β 1 gene and protein, in situ hybridization and immunohistochemistry were performed in serial sections. The first tissue section was hybridized with digoxigenin-labeled TGF- β 1 cDNA. The second and the third sections were incubated with smooth muscle cell- and macrophage-specific antibodies to identify smooth muscle cells and macrophages, respectively. The last section was used to detect TGF- β 1 protein expression. In a separate set of experiments, the expression of fibronectin, an extracellular matrix protein whose expression was found to be induced by TGF- β 1, was examined by immunohistochemistry.

In situ hybridization

A 2.0 kb EcoRI fragment of human TGF- β 1 cDNA was labelled with digoxigenin (DIG)-dUTP according to the manufacturer's instruction (Boehringer Mannheim Biochemica, Mannheim, Germany) and used as a probe for in situ hybridization, which was performed according to a published method (Sassoon and Rosenthal, 1993) as previously described (Pang et al., 1996). TGF- β 1 cDNA was isolated from a plasmid, pHTGF β -2, obtained from the American Type Culture Collection (Maryland, USA) (Ardinger et al., 1988). Arterial segments were immersion fixed in 4% paraformaldehyde buffered with 0.1M sodium phosphate (pH 7.4) for 3 h at room temperature and subsequently dehydrated in sequential 50%, 70%, 80%, 90% and absolute alcohol washes. Tissues were embedded in paraffin and prepared for in situ hybridization. Paraffin-embedded tissue sections (5-6 μ m thick) were placed on poly-L-lysine coated slides, deparaffinized, treated with proteinase K (1 μ g/ml for 15 min at 37 °C) and acetylated (0.25% acetic anhydride in 0.1M triethanolamine and 0.9% NaCl for 10 min). Sections were then washed with 2xSSC, dehydrated, air dried for 30 min and stored at -70 °C before use. Prior to hybridization, each section was prehybridized in a humid chamber with 100 μ l prehybridization solution containing 5xSSC, 5xDenhardt's solution, 50% deionized formamide, 250 μ g/ml yeast t-RNA, 250 μ g denatured salmon sperm DNA and 4 mM EDTA for 3 hr. Hybridization was performed at 50 °C for 16-24 hr in a humid chamber with 25 μ l/section prehybridization solution containing 40 ng/ μ l DNA probe. After the

hybridization, sections were washed at 42 °C twice in 2xSSC, once in 0.2xSSC and twice in 0.1xSSC for 15 min per wash. Sections were then blocked (30 min), incubated with alkaline phosphatase-conjugated anti-DIG antibody (30 min) and detected with color solution containing 337.5 μ g/ml nitroblue tetrazolium salt and 175 μ g/ml 5-bromo-4-chloro-3-indolyl-phosphate (40-60 min) according to the manufacturer's instructions (DIG labeling and detection kit, Boehringer Mannheim Biochemica). In some experiments, tissue sections were hybridized with DIG-labeled probes plus 50 fold unlabeled cDNA which abolished the signal as controls.

Immunohistochemistry analysis

For immunohistochemistry, slides were deparaffinized, rehydrated, and washed with phosphate-buffered saline. Sections were incubated with 1% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity and to permeabilize the cells. Nonspecific binding was blocked by preincubation with PBS containing either 1% normal serum or 5 mg/ml bovine serum albumin for 1 hr at room temperature. Sequentially, the second serial section was incubated with mouse anti- α -smooth muscle actin (1:400, 1A4, Sigma Chemical Co., USA) for 1 hr at 37 °C, which identifies vascular smooth muscle cells. The third serial section was incubated with mouse anti-rabbit macrophage (1:50, Ram II, Dako Corp., USA) for 1 hr at 37 °C, which react with rabbit monocyte/macrophage cell population. These two sections were then incubated with FITC-conjugated goat anti-mouse secondary antibody (1:400, Sigma Chemical Co., USA) at room temperature for 1.5 hr. Each incubation was followed by three 5-min washes in PBS. The last serial section was incubated with chicken anti-human TGF- β 1 primary antibody (1:15, R&D Systems, USA) for 1 hr at 37 °C. For tissue sections undertaken for fibronectin immunostaining, the slides were incubated with a sheep anti-rabbit fibronectin antibody (1:600, Biogenesis, USA) at 37 °C for 1 hr. The sections were then incubated with biotin-SP-conjugated IgG for 1 hr at room temperature. Antigen-antibody complexes were

localized by incubating with avidin-biotin-horseradish peroxidase complex for 1.5 hr at room temperature and by subsequent employing 0.5 mg/ml 3,3'-diaminobenzidine/0.01% hydrogen peroxide in 0.1M Tris-HCl buffer, pH 7.2 as a chromogen (Vector Lab, USA). Negative control was performed by omitting the incubation of tissue sections with primary antibody.

Results

TGF- β 1 detection by *in situ* hybridization and immunohistochemistry

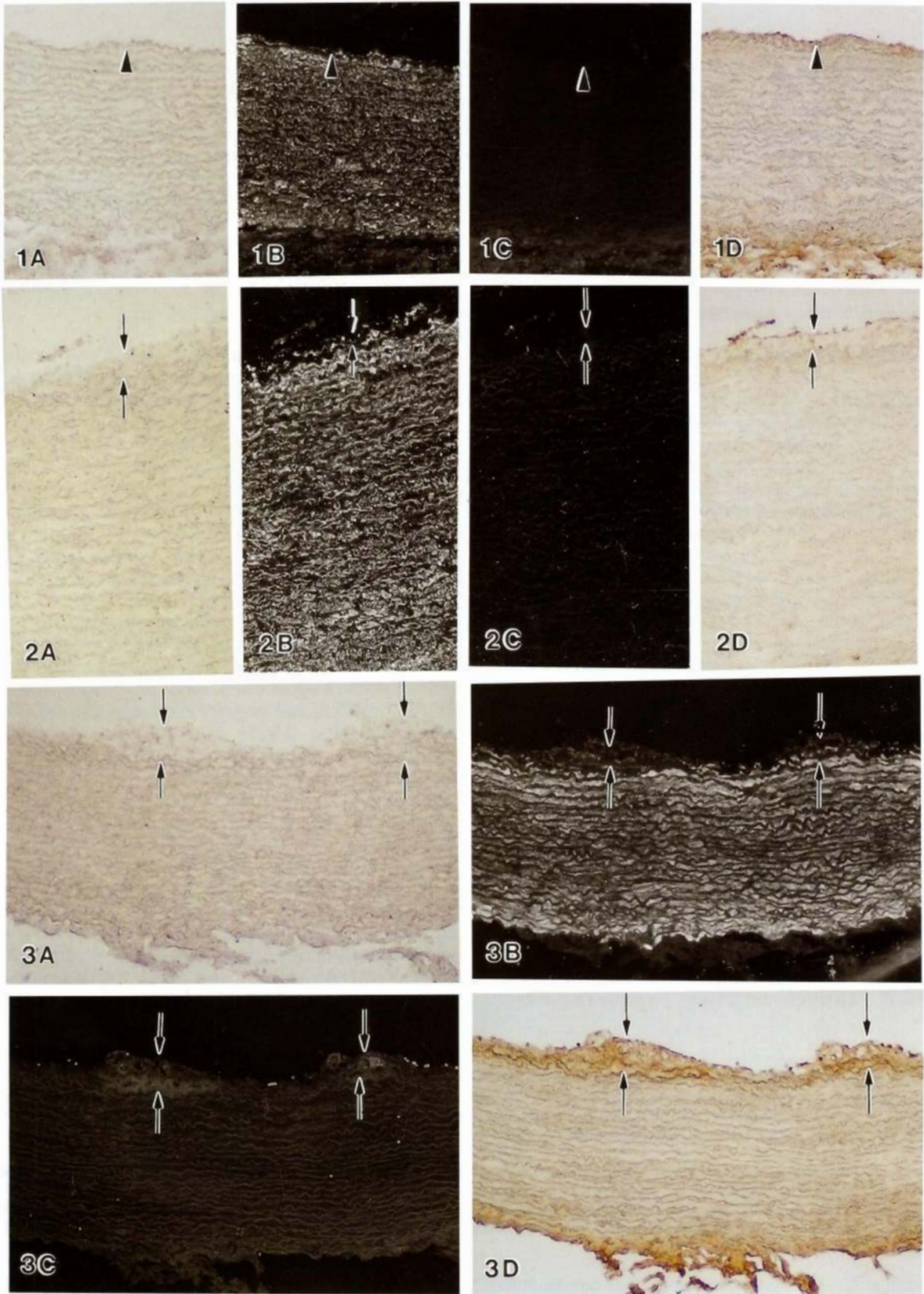
To examine the cellular expression and localization of the TGF- β 1 gene and protein during atherogenesis, *in situ* hybridization was performed in combination with immunohistochemistry. In the control group no signal for TGF- β 1 mRNA was detected (Fig. 1A). Smooth muscle cells were only detected in the tunica media (Fig. 1B) and no macrophages were apparent in the vascular wall (Fig. 1C). Results for TGF- β 1 protein staining were negative, similar to those observed for TGF β 1 mRNA (Fig. 1D). In the 1-week feeding group, no atherosclerotic lesions were detected under light microscope. The distribution of TGF- β 1 mRNA and protein, smooth muscle cells and macrophages in this group was similar to that of the control group and hence was not shown. The 2-week group exhibited slight intimal thickening in a few areas as illustrated in Fig. 2. TGF- β 1 mRNA and protein were scarcely expressed in these areas (Fig. 2A,D). Macrophage-specific staining was negative while a few smooth muscle cells were detected in the thickened intima in addition to the media (Fig. 2B,C). The 3-week feeding group exhibited small fatty streaks (Fig. 3). Parts of the intima were thickened and exhibited the TGF- β 1 mRNA and protein positive reaction (Fig. 3A,D). TGF- β 1 positive subendothelial foam cells exhibited weak immunoreactivity towards both macrophage- and smooth muscle-specific antibodies (Fig. 3C,B). The 6-week feeding group showed a markedly thickened intima which exhibited strong TGF- β 1 mRNA and protein expression (Fig. 4A,D). In contrast, TGF- β 1 mRNA and protein were not

Fig. 1. Detection of TGF- β 1 mRNA and protein in serial sections of aorta from control group. The lumen is uppermost in all sections. The internal elastic membrane is indicated by an arrowhead. **A.** *In situ* hybridization to TGF- β 1 mRNA: no signal was detected. **B.** Staining for smooth muscle-specific antibody: staining was obvious throughout the tunica media. **C.** Staining for macrophage-specific antibody: no staining was observed in the vascular wall. **D.** Staining for TGF- β 1-specific antibody: no clear staining was observed. x 130

Fig. 2. Detection of TGF- β 1 mRNA and protein in serial sections of aorta from the 2-week cholesterol-feeding group. The lumen is uppermost in all sections. The thickened intima is indicated by arrows. **A.** *In situ* hybridization to TGF- β 1 mRNA: no signal was detected in the thickened intima. **B.** Staining for smooth muscle-specific antibody: staining was clearly detected throughout the media and weak in the thickened intima. **C.** Staining for macrophage-specific antibody: no staining was observed in the whole vascular wall. **D.** Staining for TGF- β 1-specific antibody: staining was not clearly detected. x 130

Fig. 3. Detection of TGF- β 1 mRNA and protein in serial sections of aorta from the 3-week cholesterol-feeding group. The lumen is uppermost in all sections. The thickened intima is indicated by arrows. **A.** *In situ* hybridization to TGF- β 1 mRNA: weak staining was detected in the thickened intima and parts of the adventitia. **B.** Staining for smooth muscle-specific antibody: staining was clearly detected throughout the media and weak in the thickened intima. **C.** Staining for macrophage-specific antibody: staining was observed in the thickened intima. **D.** Staining for TGF- β 1-specific antibody: staining was observed in the thickened intima and parts of the adventitia. x 130

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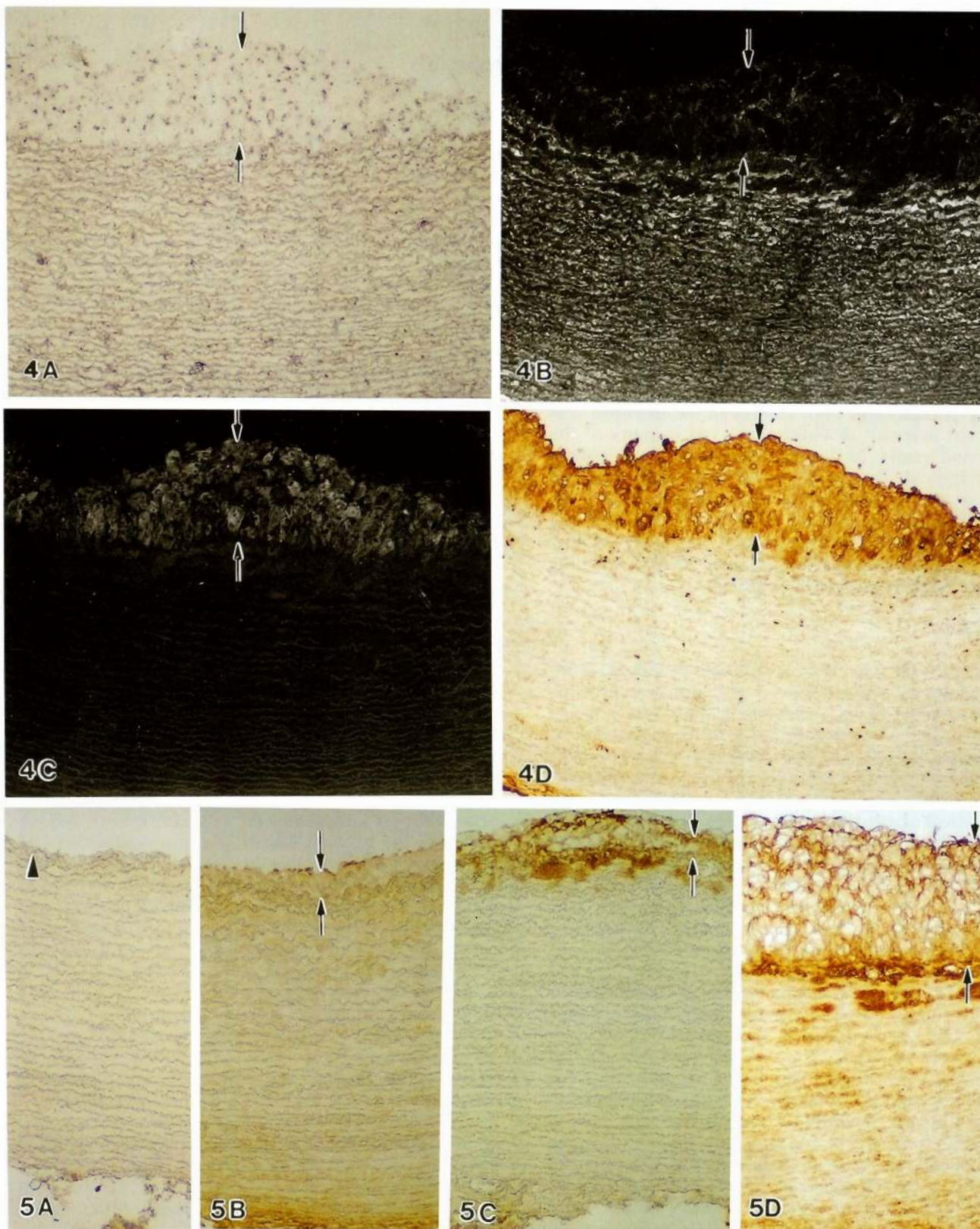


Fig. 4. Detection of TGF- β 1 mRNA and protein in serial sections of aorta from the 6-week cholesterol-feeding group. The lumen is uppermost in all sections. The thickened intima is indicated by arrows. **A.** In situ hybridization to TGF- β 1 mRNA: staining was mainly observed in the thickened intima. **B.** Staining for smooth muscle-specific antibody: staining was clearly observed throughout the media and weak in the thickened intima. **C.** Staining for macrophage-specific antibody: immunoreactivity was detected in the thickened intima. **D.** Staining for TGF- β 1-specific antibody: strong staining was observed in the thickened intima. x 130

Fig. 5. Immunostaining of fibronectin in aortic sections of control and cholesterol-feeding groups. The lumen is uppermost in all sections. The internal elastic membrane is indicated by an arrowhead and the thickened intima is indicated by arrows. Aortic sections of the control (**A**), 2-week (**B**), 3-week (**C**) and 6-week (**D**) feeding groups were reacted with fibronectin-specific antibody as described in Methods. **A.** Control: no fibronectin immunoreactivity was detected. **B.** 2-week feeding group: no staining was detected. **C.** 3-week feeding group: strong staining was detected in the proximal media and parts of fatty streaks. **D.** 6-week feeding group: strong staining was detected in the proximal media and parts of fatty streaks. x 130

detected in the underlying media of the lesioned area. The cells in the thickened intima appeared to be macrophages mainly with relatively weak immunoreactivity towards smooth muscle-specific α -actin (Fig. 4B,C).

Fibronectin detection by immunohistochemistry

The expression of fibronectin in the aortic wall during the development of atherosclerotic lesions was examined by immunohistochemistry. In the control group (Fig. 5A), 1-week (data not shown) and 2-week feeding groups (Fig. 5B), fibronectin expression was either not detected or barely detected in parts of the intima and media. In 3-week feeding group, a marked upregulation of fibronectin was detected in the proximal media as well as the thickened intima (Fig. 5C). In 6-week feeding group, an even more pronounced upregulation through the aortic wall was observed with the thickened intima and the proximal media exhibiting the highest expression levels (Fig. 5D).

Discussion

This study provides evidence demonstrating upregulation of TGF- β 1 mRNA and protein expression by macrophages as well as smooth muscle cells in the fatty streaks of hypercholesterolemic rabbits. A concomitant induction of fibronectin expression was also detected in the thickened intima and the proximal media. These results implicate a role for TGF- β 1 in the development of atherosclerotic lesions.

The New Zealand White rabbits fed with the cholesterol-containing diet are extensively used as an animal model to examine the pathogenesis and mechanisms of atherosclerosis (Jayo et al., 1994). Rabbits fed with a 2% cholesterol-containing diet for 6 weeks exhibited pronounced atherosclerotic lesions which consisted of fatty streaks. Therefore, the formation of atherosclerosis in this animal model can be easily evaluated at earlier stages within a short time period. Our previous study showed that small fatty streaks were detected in aortae after feeding the 2% cholesterol-containing diet for 3 weeks but were completely absent following one-week cholesterol feeding (Chen et al., 1999). Thus, a group with 2-week cholesterol feeding was added in this study to examine more closely the temporal correlation between the formation of fatty streaks and the expression of TGF- β 1 mRNA and protein during atherogenesis. The histological examination of aortae from the 2-week group revealed a few areas with intimal thickening in which the presence of foam cells was not apparent (Fig. 2). Thus, 2-week feeding of the 2% cholesterol-containing diet appears to be sufficient to induce intimal thickening, the stage prior to fatty streak formation in this hypercholesterolemic rabbit model.

Results from *in situ* hybridization and immunohistochemistry of serial sections indicate that TGF- β 1

mRNA and protein expression coincided with each other both temporally and spatially during atherogenesis. Up to the stage of intimal thickening observed following 2-week cholesterol feeding, TGF- β 1 mRNA and protein expression was barely detected (Fig. 2). It was after 3 weeks or 6 weeks of cholesterol feeding that the expression levels of TGF- β 1 mRNA and protein were upregulated in the thickened intima when fatty streaks became the dominant feature. In humans, the upregulation of TGF- β 1 has been reported in focal intimal thickening which predisposes those areas to the formation of more advanced atherosclerotic lesions (Stary et al., 1992; Borkowski et al., 1995; Scott et al., 1997). In contrast, our results indicate that TGF- β 1 expression is not stimulated prior to the appearance of fatty streaks, suggesting that adaptive intimal thickening is composed of a population of relatively quiescent cells in hypercholesterolemic rabbits.

Results from this study clearly demonstrate that TGF- β 1 expression is stimulated at the beginning of fatty streak formation with its expression intensity being well correlated with the development of fatty streaks. It is interesting to note that TGF- β 1 mRNA and protein expression appears to be restricted to fatty streaks following 3 weeks or 6 weeks of cholesterol feeding, no induction of TGF- β 1 was detected in the underlying media of the same groups. Those cells exhibiting increased TGF- β 1 expression appear to be macrophages mainly based on the results of cell type-specific immunoreactivity. Previous *in vitro* studies have demonstrated TGF- β 1 to be a potent chemoattractant for monocytes at subpicomolar concentrations and an activator for growth factor secretory functions of monocytes at higher concentrations (Wahl et al., 1987; Wiseman et al., 1988). The colocalization of TGF- β 1 and macrophages in fatty streaks is consistent with a role for TGF- β 1 in promoting monocyte transendothelial migration into the intima and their subsequent proliferation and activation in the thickened intima. In contrast to the clear detection for macrophages in fatty streaks, the existence of smooth muscle cells is not as prominent. These results, implying that macrophages are the primary source of TGF- β 1 during early stages of atherogenesis, point to the differential involvement of vascular cells in primary atherosclerosis and restenosis.

The upregulation of fibronectin during the development of atherosclerosis exhibits a pattern which is similar to that of TGF- β 1 temporally with a distinctive distribution spatially. An enhanced fibronectin expression was observed in the deeper layer of the thickened intima and the proximal media in the 3-week feeding group which exhibited smaller fatty streaks and less intense TGF- β 1 expression; more pronounced upregulation was detected in both intima and media of the 6-week feeding group which exhibited extensive fatty streaks with higher TGF- β 1 expression levels. While the role of TGF- β 1 as a modulator for cell proliferation is fairly complex, accumulating evidence has demonstrated TGF- β 1 to be a potent stimulator for

the synthesis of extracellular matrix including fibronectin and collagen in both normal (Nabel et al., 1993) and injured arteries (Majesky et al., 1991; Nikol et al., 1992; Wysocki et al., 1996). Indeed, stimulated extracellular matrix production was found to be the most prominent feature following in vivo expression of active TGF- β 1 in normal porcine arteries (Nabel et al., 1993). Our results indicated that both macrophages and smooth muscle cells of the fatty streaks and the medial smooth muscle are capable of upregulating fibronectin expression upon stimulation. While fibronectin was upregulated in both fatty streaks and the media, medial smooth muscle cells proximal to the internal elastic lamina exhibited the most pronounced upregulation. These results are comparable to a previous report showing colocalization between non-foamy macrophages and fibronectin mRNA expression by smooth muscle cells in the intima of human atherosclerotic pulmonary arteries (Liptay et al., 1993). Fibronectin has been implicated in the transition of smooth muscle cells from the contractile phenotype to the synthetic phenotype both in culture and in a vascular injury model (Thyberg, 1998). It is conceivable that increases in fibronectin expression in the thickened intima and the proximal media could promote the synthetic phenotype of smooth muscle cells and the subsequent migration and proliferation.

In summary, this study demonstrated that TGF- β 1 mRNA and protein expression is stimulated in macrophages and smooth muscle cells of aortic fatty streaks in hypercholesterolemic rabbits. A concomitant upregulation of fibronectin expression was also detected in the thickened intima and the proximal media. These results implicate a role for TGF- β 1 in the trans-endothelial migration of circulating monocytes, the activation of intimal macrophages and smooth muscle cells, and the stimulation of extracellular matrix synthesis by smooth muscle cells during the development of atherosclerotic lesions.

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