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ORIGINAL ARTICLE

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Expression of platelet-derived growth factor and c-*myc* in atherosclerotic lesions in cholesterol-fed chickens: immunohistochemical and in situ hybridization study

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Abstract Immunohistochemical examination showed no significant expression of platelet-derived growth factor-A (PDGF-A), PDGF-B, PDGF receptors, or of c-*myc* in the thoracic and abdominal aortas of normal roosters. In cholesterol-fed roosters, intense immunohistochemical reaction for PDGF-B, PDGF receptor, and c-*myc* was seen in the lipid-rich thickened intimal lesions of the thoracic and abdominal aortas while no significant immunoreaction for PDGF-A was demonstrated in the same lesions. In accordance with immunohistochemical findings, in situ hybridization demonstrated a significant level of expression of PDGF-B, PDGF-A receptor, PDGF-B receptor, and c-*myc* genes in proliferating intimal cells of the thoracic and abdominal aortas. These results suggest that coordinate actions of PDGF-B and c-*myc* play an important role in proliferation of intimal cells in the developing atherosclerotic lesions in chickens.

Key words PDGF · PDGF-receptor · c-*myc*
Atherosclerosis · Rooster

Introduction

Proliferation of arterial intimal smooth muscle cells has been recognized as one of the key cellular events in the development of atherosclerotic lesions [5, 28]. Ross [28] suggested that chronic cycles of injury and repair are responsible for the development of atherosclerosis, and that injury is exacerbated by risk factors for atherosclerosis such as hypertension, hypercholesterolaemia, and smoking cigarettes. Among numerous growth factors playing important roles in atherogenesis, platelet-derived growth factor (PDGF) has been considered to be a potent mitogen for mesenchymal cells such as smooth muscle cells and glial cells [9, 29]. The monoclonal hypothesis proposed by Benditt and Benditt [5] suggested that atherosclerotic plaques represent a benign neoplasm originated from a virally-infected or chemically-injured cells. Penn et al. [24] identified a transforming gene different from v-Ki-*ras*, N-*ras* and v-Ha-*ras* genes, in human atherosclerotic plaque DNA. We have previously reported that several oncogene products are expressed in human coronary atherosclerotic lesions [33]. Thus, the present study was carried out to examine the role of PDGF and c-*myc* in the development of atherosclerotic lesions in chickens, which serve as a useful experimental model for the study of atherosclerosis [32].

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Materials and methods

Five 4-month-old normal roosters and six age-matched cholesterol-fed roosters were used in the present study. Six 1-month-old cockerels were fed an atherogenic diet containing 2% cholesterol and 10% corn oil for 3 months. The thoracic and abdominal aortas were collected, fixed in a 10% formalin solution and embedded in paraffin. Paraffin sections 4 μm thick were cut and stained with haematoxylin and eosin, Mallory azan, and elastic van Gieson. Immunostaining was performed on tissue sections by the avidin-biotin-peroxidase complex (Vector, Burlingame, Calif., USA) method [13]. Sections were treated with 0.3% hydrogen peroxide to block endogenous peroxidase. After exposure to non-immune serum, the sections were reacted with either the primary anti-sera or non-immune sera, link biotinylated antibodies, followed by avidin-peroxidase complex at room temperature. The sections were washed three times with 0.1 M phosphate buffered saline (pH 7.5) for 5 min after each antibody application, and then treated with diaminobendizine in hydrogen peroxide. Primary antibodies raised against alpha-smooth muscle actin, OKM1, vimentin (DAKO), PDGF-A, PDGF-B(Genzyme), PDGF receptor, and pan-*myc* (Cambridge Research Biochemicals) were used in the present study. Serum cholesterol was determined by the enzymatic method (TC-S 736, Kyowa, Tokyo).

In situ hybridization was carried out according to the method reported by Cox et al. [8]. Glass slides were prewashed in chromic acid mixture and coated with poly L-lysine. Sections 4 μm thick were mounted on the treated slides, dried at 40° C and deparaffini-

zed. Specimens on the slides were digested with proteinase K [1 μg/ml in 100 mM TRIS-hydrochloric acid (HCl), 50 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0] for 30 min at 37° C. The slides were then treated with 0.25% (v/v) acetic anhydride and 0.1 M triethanolamine, pH 8.0, for 10 min, and then dehydrated in increasing concentrations of ethanol. Probes for detecting the PDGF-A, PDGF-B, PDGF-A receptor, and PDGF-B receptor mRNAs were purchased from Oncogene Science, and were biotinylated using a terminal labelling kit (Enzo Biochem). The probe for c-*myc* mRNA was prepared by inserting the Sma I fragment (exon II) of pMyc6514 (a gift from the Japanese Cancer Research Resources Bank) into plasmid pSP65. Biotinylated RNA probes were synthesized in vitro according to a modification of the method of Melton et al. [19], using SP6 RNA polymerase, linearized plasmid DNA and biotin 11-UTP. RNA probes were dissolved in 50% formamide and 10mM dithiothreiotol (DTT) and then diluted with 4 volumes of hybridization buffer [0.3 M sodium chloride (NaCl), 20 mM TRIS-HCl, pH 8.0, 5 mM EDTA, 0.02% Ficoll 400, 0.02% polyvinyl pyrolidone, 0.02% bovine serum albumin fraction V, 50% formamide, 10% dextran sulphate, 1 mg/ml yeast tRNA and 10 mM DTT]. Specimens on slides were first treated with 50% formamide solution (same as the hybridization buffer except for 10% dextran sulphate and yeast RNA) at 37° C for 2 h. After removing the 50% formamide solution, 60 μl of the probe solution was applied to each slide, which were then incubated at 37° C for 30 h in a moist chamber. The slide was then washed once with SSC, treated with RNase A solution (20 μg/ml in 0.5 M NaCl, 10 mM TRIS-HCl, pH 8.0, and 1 mM EDTA) at 37° C for 30 min, and washed finally in 0.1×SSC. Biotinylated probes hybridized on slides were visualized with Blu Gene (Bethesda Research Laboratories, Life Technologies, Inc.).

Results

Serum levels of cholesterol ranged from 137 to 193 mg/dl in normal 4-month-old roosters, and from 655 to 1,806 mg/dl in those fed cholesterol.

Histologically 4-month-old normal roosters showed characteristic medial architecture with alternating layers of smooth muscle cells and fibroblasts in the thoracic aorta. In this area, intimal thickening was rarely seen (Fig. 1A). The abdominal aorta occasionally showed slight intimal thickening composed of smooth muscle cells (Fig. 1B). Cholesterol-fed 4-month-old roosters displayed lipid-rich intimal thickening in both thoracic and abdominal aortas, but to a greater degree in the former than in the latter. This lipid deposition extended to the inner layer of the tunica media in the thoracic aorta (Fig. 2A). Compared with the abdominal aortas from normal roosters, those from cholesterol-fed roosters showed more significant intimal thickening (Fig. 2B). Lipid-containing cells were also seen in the superficial layer of the thickened intima.

Immunoreaction of alpha-smooth muscle actin was observed in alternating layers of smooth muscle cells of the thoracic aorta and in the thickened intima of the abdominal aorta (Fig. 3) from both normal and cholesterol-

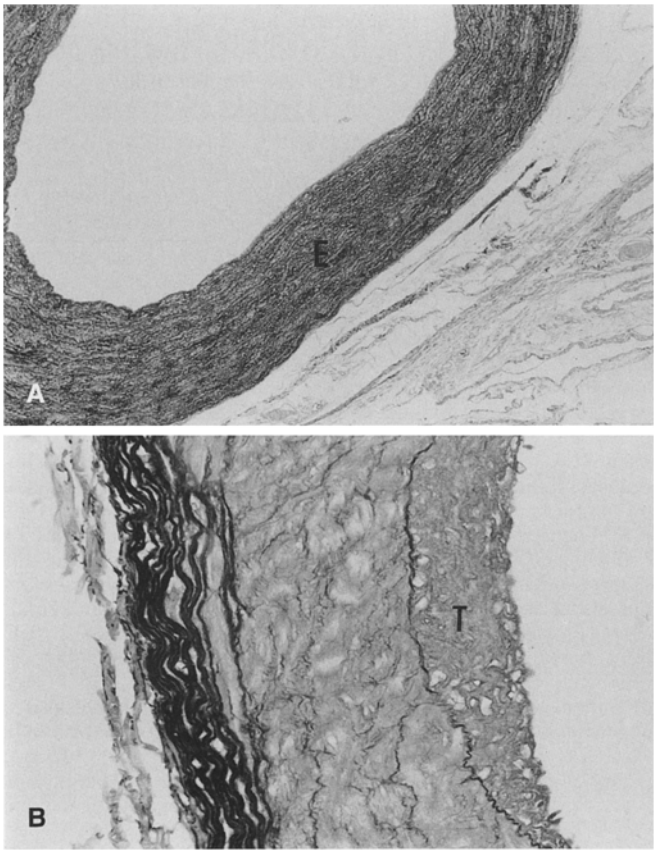

Fig. 1A, B Histological architecture of the aortas from a normal rooster. **A** Multilayered elastic lamellar structure (*E*) of the thoracic aorta [elastic van Gieson (EVG), ×30]. **B** Abdominal aorta with slight intimal thickening (*T*; EVG ×300)

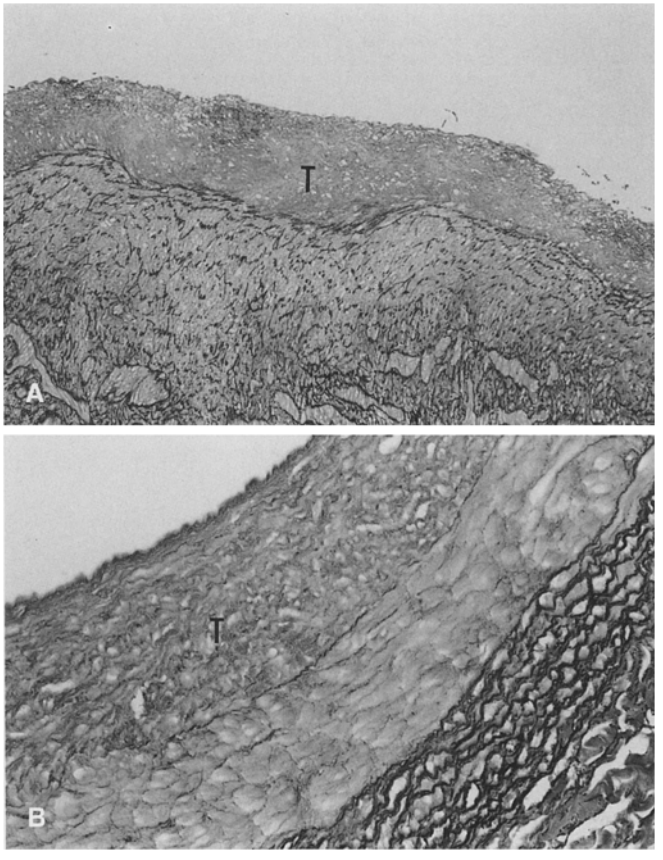

Fig. 2A, B Marked lipid-rich intimal thickening (*T*) of the aortas from a cholesterol-fed rooster. **A** Thoracic aorta (EVG ×75). **B** Abdominal aorta (EVG ×150)

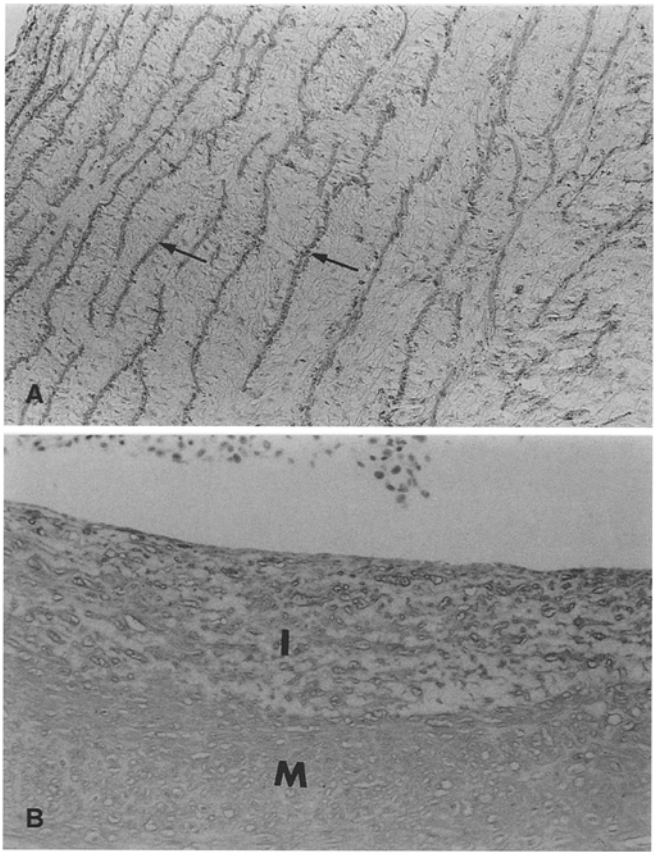

Fig. 3A, B

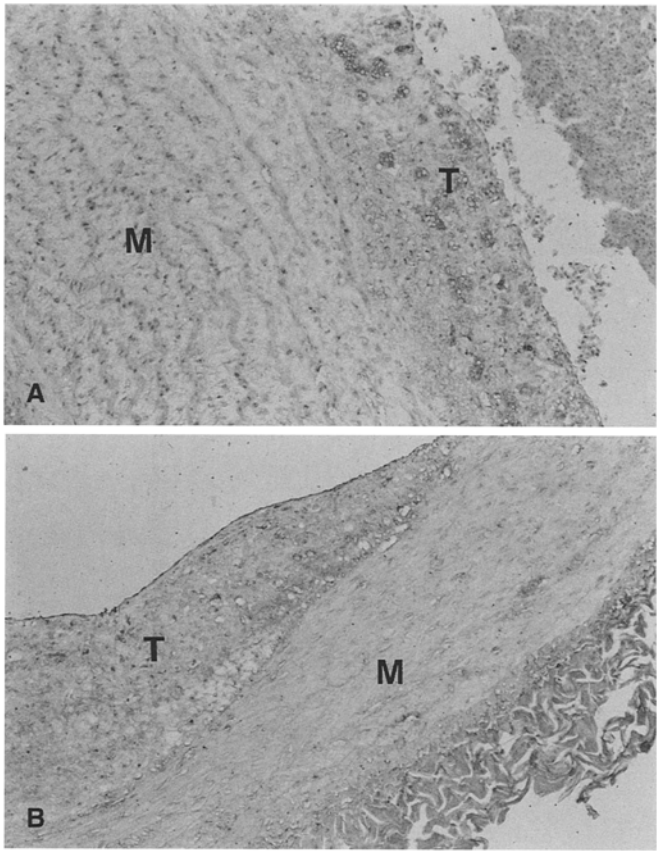

Fig. 5A, B Immunohistochemical reaction for vimentin was more positively seen in the thickened intima (*T*) than the tunica media (ABC method). **A** Thoracic aorta (×150). **B** Abdominal aorta (×150)

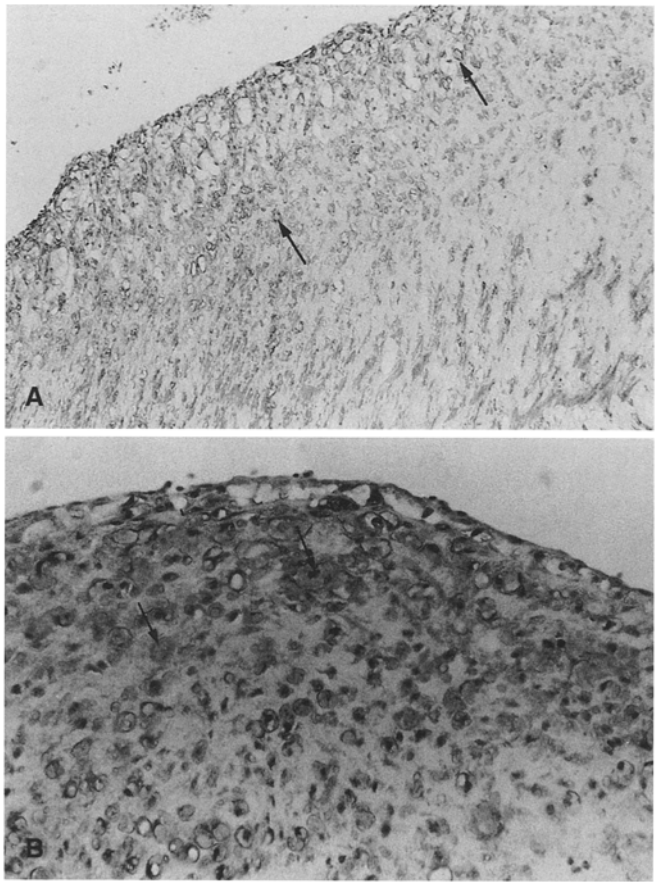

Fig. 4A, B

fed roosters. In normal roosters, no significant immunostaining for OKM1, PDGF-A, PDGF-B, PDGF receptor, or c-*myc* was observed in the thoracic or abdominal aortas, whereas weak vimentin immunostaining was occasionally seen in the thoracic and abdominal aortas. In cholesterol-fed roosters, various immunohistochemical reactions with these antibodies were observed in the thoracic and abdominal aortas. OKM1 immunoreaction was demonstrated in the thickened intima of the thoracic and abdominal aortas, but the frequency of OKM1-positive cells was higher in the thickened intima of the thoracic aorta than in that of the abdominal aorta (Fig. 4). Positive vimentin immunoreaction was seen in proliferating intimal cells of both thoracic and abdominal aortas (Fig. 5). Immunohistochemical reaction with the anti-PDGF-A serum was weak or negative in the thickened intima of

Fig. 3A, B Positive immunoreaction for alpha-smooth muscle actin was demonstrated in smooth mucle cells [avidin-biotin-peroxidase complex (ABC) method]. **A** Multilayered medial smooth muscle cells (*arrow*) of the thoracic aorta from a normal rooster (×150). **B** Intimal (*I*) and medial (*M*) smooth muscle cells in the abdominal aorta from a cholesterol-fed rooster (×150)

Fig. 4A, B Immunohistochemical staining for OKM1 (*arrow*) in intimal thickening lesions (ABC method). **A** Thoracic aorta (×150). **B** Abdominal aorta (×340)

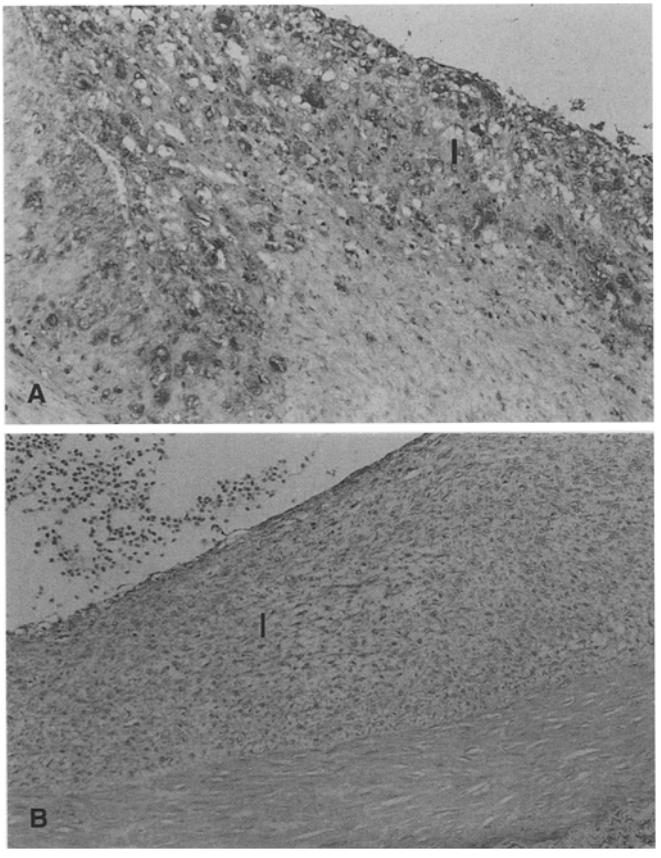

Fig. 6A, B

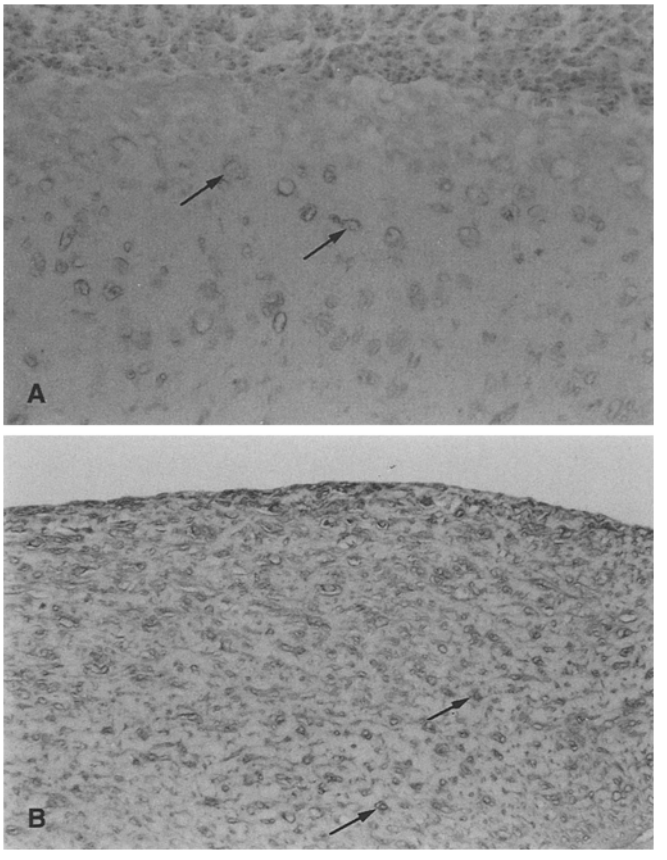

Fig. 8A, B Immunohistochemical staining of c-*myc* oncoprotein (*arrow*) in the intimal thickening lesion (ABC method). **A** Thoracic aorta (×170). **B** Abdominal aorta (×300)

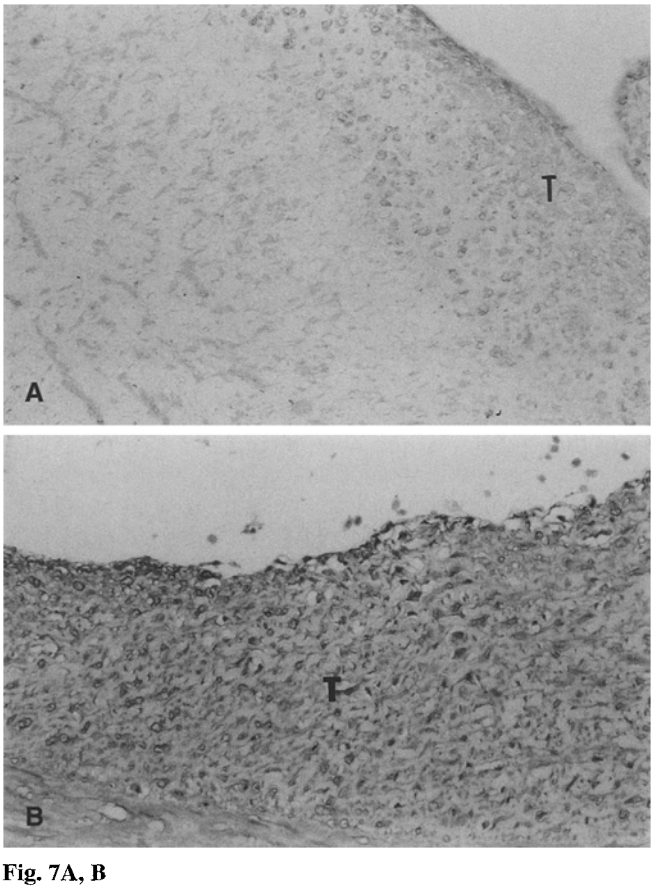

Fig. 7A, B

the thoracic and abdominal aortas from cholesterol-fed roosters. Intense immunostaining for PDGF-B was observed in lipid-containing intimal cells in the thoracic and abdominal (Fig. 6) aortas, and immunostaining patterns for PDGF receptor (Fig. 7) and c-*myc* (Fig. 8) correlated well with that for PDGF-B.

Correlating well with the immunohistochemical results, in situ hybridization demonstrated no significant signals for transcripts of PDGF-A, PDGF-B, PDGF-A receptor, PDGF-B receptor, or c-*myc* genes in both thoracic and abdominal aortas from normal roosters. PDGF-B mRNA was detected in both lipid-rich intimal lesions of the thoracic aorta and in those of the abdominal aorta (Fig. 9). Although PDGF-A receptor transcript signal was weak, that of PDGF-B receptor was demonstrated in proliferating intimal cells of the thoracic and abdominal aortas (Fig. 10). Positive signals for c-*myc* messenger RNA were also demonstrated in proliferating intimal cells in both thoracic and abdominal aortas (Fig. 11).

Fig. 6A, B Platelet-derived growth factor (PDGF)-B is positively stained in proliferating intimal cells (*I*; ABC method). **A** Thoracic aorta (×150). **B** Abdominal aorta (×170)

Fig. 7A, B Note positive immunohistochemical reaction for PDGF receptor in the thickened intima (*T*; ABC method). **A** Thoracic aorta (×150). **B** Abdominal aorta (×300)

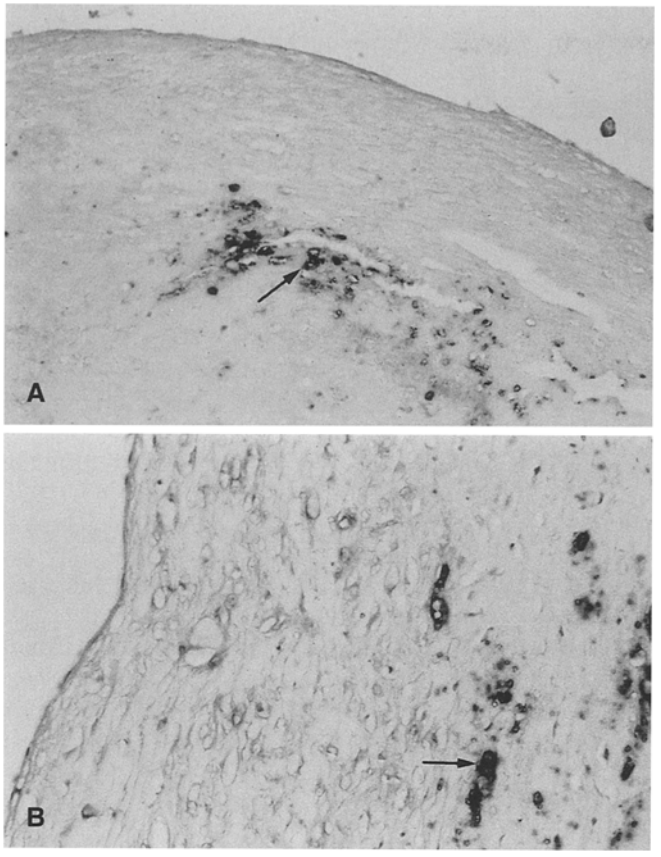

Fig. 9A, B

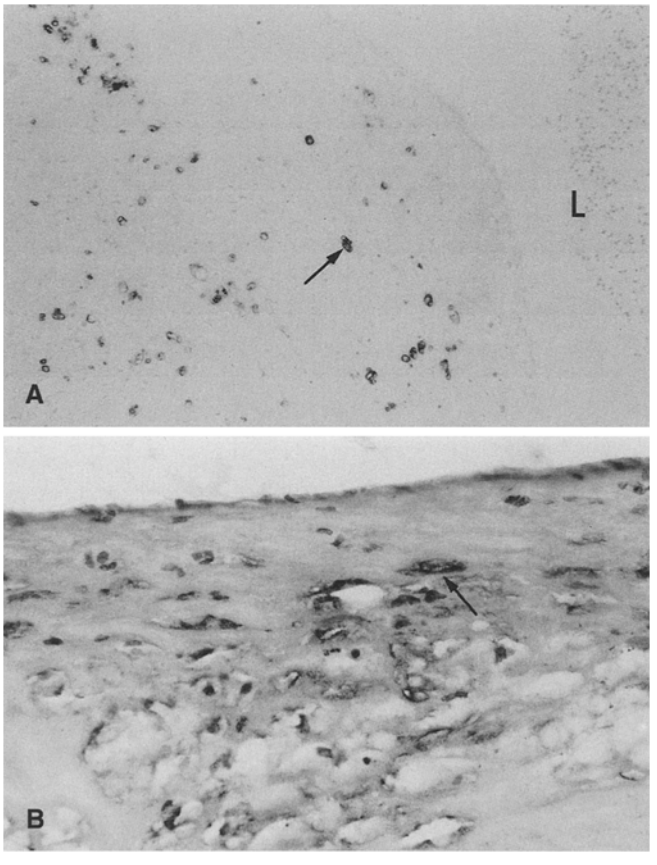

Fig. 10A, B

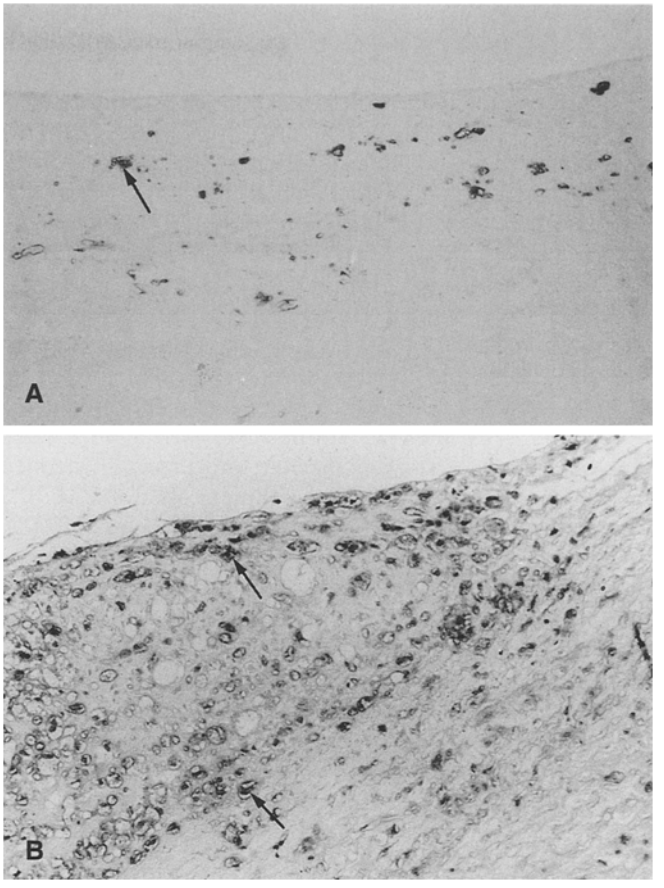

Fig. 11A, B Signal expression of c-*myc* gene mRNA (*arrow*; in situ hybridization). **A** Thoracic aorta (×150). **B** Abdominal aorta (×170)

Discussion

The present study has clearly demonstrated that lipid-rich intimal thickening lesions are more prevalent in the thoracic than in the abdominal aorta. We have previously shown that the main cell types of proliferating intimal cells were fibroblasts and smooth muscle cells in the thoracic aorta and abdominal aorta, respectively [32]. In the present study, most lipid-containing cells in the thickened intima of the thoracic aorta reacted with OKM1, a marker of monocytes and leukocytes [3]. Inaba et al. [14] reported that cultured smooth muscle cells express c-*fms* and the scavenger receptor which is considered to be specific for macrophages. Although it is sometimes difficult to differentiate lipid-containing fibroblasts and macrophages, we speculate that proliferating intimal cells are composed for both of these cell types. Most proliferating intimal cells in the thoracic aorta also reacted with the antibody raised against vimentin. Gabbiani et al. [12] re-

Fig. 9A, B Signal expression of c-*sis* mRNA (*arrow*; in situ hybridization). **A** Thoracic aorta (×150). **B** Abdominal aorta (×300)

Fig. 10A, B Signal expression of PDGF-B receptor mRNA (*arrow*; in situ hybridization; *L:* lumen). **A** Thoracic aorta (×150). **B** Abdominal aorta (×300)

ported that vimentin-rich cells contribute to aortic intimal thickening after experimental mechanical endothelial injury in rats. Positive immunoreaction for vimentin was demonstrated in proliferating intimal fibroblasts in the thoracic aorta and intimal smooth muscle cells in the abdominal aorta in cholesterol-fed roosters. Therefore, the present immunohistochemical results suggest that phenotypical transformation of fibroblasts and smooth muscle cells is characteristic of the development of atherosclerotic lesions in chickens.

PDGF is a dimer composed of two polypeptide chains, A and B [6, 27]. The A chain gene maps to chromosome 22 [6] and contains seven exons spanning 22 kb of genomic DNA [27]. PDGF A-chain has been detected in various cells such as gliomas [2], melanomas [21], and fibroblasts [23]. The B-chain precursor is encoded by the c-*sis* gene, which is the cellular counterpart of the transforming gene v-*sis* of simian sarcoma virus [4, 15]. The c-*sis* gene has been shown to be transcribed in endothelial cells [16], placental cytotrophoblasts [7] and active macrophages [18]. Martinet et al. [18] demonstrated that activated human monocytes express the c-*sis* protooncogene and release a mediator showing PDGF-like activity. Wilcox et al. [34] reported that PDGF-A is expressed in smooth muscle cells and PDGF-B is expressed in macrophages in atherosclerotic lesions, and Ross et al. [30] showed that PDGF-B protein was localized within macrophages in all stages of atherosclerotic lesions in both human and nonhuman primates. They speculated that the entry of monocytes into the arteries and their differentiation into macrophages may stimulate the expression of PDGF-B gene, following decreased expression of PDGF-B gene in lipid-rich foam cells. However, the results of the present study have demonstrated that numerous lipid-containing intimal fibroblasts and macrophages significantly expressed the PDGF-B gene, but not PDGF-A. Therefore, we surmise that active lipid-containing intimal cells such as fibroblasts and macrophages are responsible for production of PDGF-B, rather than fibroblasts without lipid droplets or degenerating lipid-containing fibroblasts.

It has been shown that alpha- and beta-receptors are responsible for the cellular effects of PDGF [31], and these two receptors have been reported to exist on cultured smooth muscle cells [25]. The present immunohistochemical and in situ hybridization studies suggested that PDGF-A and PDGF-B receptors are expressed in intimal cells such as macrophages, fibroblasts and smooth muscle cells when these activated intimal cells transform into lipid-containing cells.

Myc oncogene has been reported to possess a regulatory function in a variety of cell types and tissues, and to be rearranged, amplified, and overexpressed in a wide variety of human cancers [1, 10, 26]. Several previous reports have suggested that protooncogenes such as c-*myc* and c-*fos* may be involved in the mechanisms of atherosclerosis [17, 22]. Komuro et al. [17] reported that endothelin caused a rapid and transient increase in c-*myc* mRNA levels and stimulated DNA synthesis in vascular smooth muscle cells. Parkes et al. [22] reported that expression of c-*myc* by human plaque smooth muscle cells was also enhanced, when compared with smooth muscle cells derived from healthy human aorta and saphenous vein. Frick and Scher [11] reported that PDGF regulates c-*myc* expression in many cell types. In the present study, we have demonstrated that the c-*myc* gene was expressed in lipid-rich intimal lesions, especially those of thoracic aorta, but not in the normal aortic wall. These results suggest that expression of c-*myc* gene is actively involved in the proliferation of intimal cells such as fibroblasts and macrophages which are related to the development of atherosclerotic lesions in the thoracic aorta in chickens. Moss and Benditt [20] proposed the monoclonal hypothesis of atherogenesis from ultrastructural observations of abdominal plaques which have different cellular components and histogenesis from intimal thickening of the thoracic aorta and its large branches. We have shown that PDGF and c-*myc* were significantly expressed in the plaques in the abdominal aorta, but were less intense than those of the thoracic aorta in cholesterol-fed roosters. Therefore, we believe that these plaques formed in normal roosters by haemodynamic stress are essential for the development of the atherosclerotic plaques in cholesterol-fed roosters, and that growth factors such as PDGF stimulate proliferation of plaque smooth muscle cells through activation of nuclear-related genes such as c-*myc*.

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