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OSTEOPONTIN EXPRESSION IN SPONTANEOUSLY DEVELOPED NEOINTIMA IN FOWL (*GALLUS GALLUS*)

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Summary

Fowl show spontaneous elevation of blood pressure and neointimal plaque formation in the abdominal aorta at young ages. A similar neointima can be induced by a balloon-catheter-induced endothelium injury to the fowl aorta. Both spontaneously developed and injury-induced vascular lesions exhibit subendothelial hyperplasia consisting of neointimal cells with a synthetic phenotype and abundant extracellular matrix. The role of the extracellular matrix in the formation of neointima is not known. In this study, we investigated whether osteopontin, an adhesive glycoprotein present in the extracellular matrix, is expressed in aortic smooth muscle tissue of the fowl abdominal aorta, in spontaneously developed neointimal plaques and in the aortic smooth muscle underlying neointimal plaques. Crude protein extracted from isolated aortic smooth muscle tissues and neointimal plaques was fractionated by SDS–polyacrylamide gel electrophoresis and analyzed by immunoblotting with rabbit anti-fowl osteopontin (provided by Dr L. C. Gerstenfeld, Boston University) or anti-alpha smooth muscle actin antibodies. The anti-fowl osteopontin antibody predominantly recognized a 66–70 kDa protein band in neointimal plaques that co-migrated with the osteopontin phosphoprotein from chick bone. In contrast, intact aortic smooth muscle and the smooth muscle underlying neointimal plaques equally expressed three proteins (66–70 kDa, approximately 50 kDa and approximately 43 kDa) recognized by the anti-osteopontin antibody. Anti-alpha smooth muscle actin antibody recognized a 43 kDa protein band, and the expression of alpha smooth muscle actin was higher in aortic smooth muscle than in neointimal plaques. Osteopontin mRNA expression was examined using reverse transcription–polymerase chain reaction (RT–PCR) of total RNA from vascular tissues with specific primers constructed on the basis of the reported fowl osteopontin nucleotide sequence. The PCR products from intact aortic smooth muscle and neointimal plaques correspond to the product from recombinant plasmid cDNA (a gift from Dr L. C. Gerstenfeld) transcribed *in vitro*. These results suggest that osteopontin is synthesized in intact aortic smooth muscle and neointimal plaques in fowl and that unmetabolized approximately 66 kDa osteopontin protein is a predominant form in the neointima, indicating that osteopontin protein may be actively synthesized in the neointima.

Key words: osteopontin, vascular smooth muscle, neointimal plaque, fowl, *Gallus gallus*, vascular lesion, alpha smooth muscle actin.

Introduction

Mammalian arteries form neointimal lesions in response to various types of vascular injuries, including radiation, mechanical injury, electrical stimulation, embolectomy and wrapping of the vessels (Ross, 1993; Schwartz et al., 1995). Many avian species spontaneously develop high blood pressure and form vascular lesions of the aorta and large arteries that partly resemble atherosclerosis (Grollman et al., 1963; Moss and Benditt, 1970; Rymaszewski et al., 1976; Gupta and Grewal, 1980; Nishimura et al., 1981; Kamimura et al., 1995). Neointimal plaques can also be experimentally induced in fowl by causing endothelium injury in the aorta and ischiadic arteries with an embolectomy catheter (Madison and Nishimura, 1994; A. B. Madison and H. Nishimura, unpublished observations). Both spontaneously developed and endothelium-injury-induced neointimal plaques contain neointimal cells of the synthetic phenotype and abundant extracellular matrix (Madison and Nishimura, 1994; Qin and Nishimura, 1998).

Giachelli et al. (1993, 1995) reported that rat neointimal plaques, induced in the aorta or carotid artery by a balloon catheter, and human atherosclerotic plaques show substantial expression of osteopontin, an extracellular matrix protein and a ligand for cell surface integrins (Liaw et al., 1995). Osteopontin is an acidic glycosylated phosphoprotein of

approximately 41.5 kDa (Butler, 1989) that specifically binds Ca^{2+} (Singh et al., 1993). Osteopontin has been isolated from rat, human and bovine bones (for reviews, see Butler, 1989; Denhardt and Guo, 1993) and appears to play an important role in bone mineralization (Butler, 1989; Denhardt and Guo, 1993), in cell adhesion and migration (Clyman et al., 1992; Liaw et al., 1994) and possibly in downregulation of nitric oxide production (Denhardt and Guo, 1993). In fowl, a 66 kDa phosphoprotein (Gotoh et al., 1990) and cDNA (Moore et al., 1991) have been isolated, respectively, from cultured chick embryonic osteoblasts and embryonic chicken bones; the phosphoprotein was later characterized and designated an avian homologue to mammalian osteopontin (Moore et al., 1991; Gotoh et al., 1995). No study was made, however, of whether fowl blood vessels express osteopontin. We sought to determine whether osteopontin protein and mRNA are expressed in fowl abdominal aortic smooth muscle tissues and whether similar levels of osteopontin expression are present in spontaneously developed neointimal plaques.

Materials and methods

Animals and maintenance

One-day-old female chicks of domestic fowl *Gallus gallus* (White Leghorn breed, Delta strain) were purchased from Heartland Hatchery (Portland, IN, USA) or DeKalb Ozark (Neshoba, MO, USA). Chicks were maintained in temperature-controlled (37 °C, then gradually reduced) brooders for 3–4 weeks and thereafter kept in groups (5–25 birds per cage) in large indoor pens (1.10 m×1.02 m×1.88 m; length×width×height) with raised plastic mesh floors. Temperature (22–24 °C) and photoperiod (12 h:12 h light:dark cycle) were controlled. Chicks and pullets under 20 weeks of age were fed Start & Grow (17 % protein, 0.75 % calcium; Purina; St Louis, MO, USA) with drinking water *ad libitum* supplemented with vitamins (Vita-Start; G.Q.F. Manufacturing Co.; Savannah, GA, USA) for 3 weeks. Adult chickens (over 21 weeks of age) were fed Layena (16 % protein, 3.6 % calcium; Purina).

Reagents

Avian Ringer's solution (referred to as Ringer) consists of (in mmol l^{-1}) 115 NaCl, 5.0 KCl, 0.5 $MgCl_2$, 6 H_2O, 0.8 Na_2HPO_4, 0.2 $NaH_2PO_4 \cdot H_2O$, 25 $NaHCO_3$, 8.3 glucose, 5 alanine and 10 sodium acetate, pH 7.4. Coomassie Blue R-250, Tween-20 (polyoxyethylene sorbitan monolaurate), polyacrylamide and bis-acrylamide were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Seakem GTG agarose was purchased from FMC BioProducts (Rockland, ME, USA). Random primers, Superscript II reverse transcriptase, dithiothreitol and *Taq* DNA polymerase were obtained from Life Technologies (Rockville, MD, USA); dNTPs were obtained from Promega Corp. (Madison, WI, USA). Guanidine thiocyanate, beta-mercaptoethanol, sodium lauryl sarcosinate, cesium chloride, EDTA, diethyl pyrocarbonate (DEPC), formaldehyde, methyl alcohol, phenylmethylsulfonyl fluoride (PMSF), Tris, SDS and general reagents were from Sigma Chemical Co. (St Louis, MO, USA).

Isolation of aortic tissue and extraction of total protein

Birds were quickly killed by decapitation, and the entire aorta was removed and placed in Ringer with moderate air flow. The aorta was cut open and examined under a dissecting microscope. If present, neointimal plaques were removed with fine forceps and placed in Ringer. Vascular plaques are most frequently found in the distal segment of the abdominal aorta immediately above the ischiadic bifurcation. With advancing age, plaques are seen more diffusely in the abdominal aorta and its branching points. Aortic smooth muscle layers from the distal segment of the abdominal aorta (up to 1.5–2.0 cm above the bifurcation) that exhibited plaque formation (referred to as aortic smooth muscle underlying plaque) or aortic smooth muscle from aorta that contained no macroscopically visible plaques (up to 1.5–2.0 cm above the bifurcation, referred to as intact aortic smooth muscle) were isolated from the adventitia in Ringer containing 50 mmol l^{-1} mannitol. A slightly hypertonic solution helps to isolate smooth muscle tissue quickly (Qin and Nishimura, 1998); mannitol was completely removed and had no influence on smooth muscle function (Qin and Nishimura, 1998). Tissues were washed in 1.0 ml of Tris-buffered saline (pH 7.4) that contained (in mmol l^{-1}) 155 NaCl, 10 Tris-HCl, 0.5 EDTA and 5 PMSF. Tissues were then homogenized with a Polytron homogenizer (Brinkman; Westbury, NY, USA) on ice four times (dial setting 8) at 10 s intervals in a buffer (pH 7.4) containing 2 % SDS plus (in mmol l^{-1}) 100 Tris-HCl, 5.0 EDTA and 0.1 PMSF. The tissues from 4–6 birds from the same age group were pooled for one determination. The number of determinations (n) and the number of birds (N) used for each protocol are indicated in the Results section. The birds from which intact aortic smooth muscle was collected were generally younger than those used for collection of neointimal plaques and aortic smooth muscle underlying neointimal plaques.

Total protein was extracted from neointimal plaques and aortic smooth muscle tissues by a modification of the method of Tsukada et al. (1987). Briefly, the homogenate was boiled for 10 min and then centrifuged at 5000 ***g*** for 15 min (4 °C). The supernatant of crude tissue extract was incubated overnight at −70 °C with 10 % (v/v) methanol (Sigma Chemical Co.) to concentrate the protein fraction and to remove excess SDS, low-molecular-mass contaminants and lipids. The supernatant (protein fraction) was collected by centrifugation at 13 000 ***g*** for 15 min at 4 °C, and the protein concentration of the supernatant was determined using the method of Lowry et al. (1951).

SDS–polyacrylamide gel electrophoresis and western immunoblot analysis

Total proteins from spontaneously developed neointimal plaques, the aortic smooth muscle underlying plaques or intact aortic smooth muscle tissues were electrophoresed on a 0.1 %

SDS/12 % polyacrylamide gel (Laemmli, 1970) with Tris-glycine buffer (pH 8.3; Bio-Rad Laboratories) containing (in mmol l^{-1}) 25 Tris-HCl, 192 glycine and 1 % SDS. After electrophoresis, gels were either stained with Coomassie Blue (0.25 % in 40 % methanol and 10 % glacial acetic acid for 1–3 h, destaining as needed) or transferred to nitrocellulose membranes (Gibco-BRL Life Technologies; Gaithersburg, MD, USA) using the above-mentioned Tris-glycine buffer (pH 8.3) containing 20 % (v/v) methanol and no SDS. For immunoblotting, the nitrocellulose sheets were first blocked with 3 % gelatin (Bio-Rad) in Tris-buffered saline (20 mmol l^{-1} Tris, 500 mmol l^{-1} NaCl containing 0.05 % Tween-20, referred to as TTBS, pH 7.5, heated to 50 °C) for 1 h at ambient temperature (21–23 °C) and then, following a wash, incubated overnight at ambient temperature with either polyclonal rabbit anti-fowl osteopontin antibody (1:750 dilution) (a gift from Dr L. C. Gerstenfeld; Boston, MA, USA; Gerstenfeld et al., 1990) or monoclonal anti-alpha smooth muscle actin (mouse IgG2a isotype, clone 1A4; 1:1000 dilution; Sigma Chemical Co.). The nitrocellulose was washed in TTBS to remove unbound first antibody, and blots were incubated with either anti-rabbit IgG (for osteopontin) or anti-mouse IgG (for alpha smooth muscle actin) antibodies conjugated with horseradish peroxidase (1:6000, product A0545 or A2304, respectively, Sigma Chemical Co.) for 2 h at ambient temperature. Protein bands were detected by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL, USA) by incubation in equal parts of luminol/enhancer solution and stable peroxide solution. Images of the blots were then formed on Kodak scientific imaging film (X-Omat Blue XB-1; exposure 30 s to 3 min). Immunodetection by goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad alkaline phosphatase substrate kit), followed by 5-bromo-4-chloro-3 indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) solutions for color detection, was used for some studies. The molecular size of the protein was determined by comparison with SDS–PAGE marker, kaleidoscope prestained standard (Bio-Rad).

Two interassay controls were used: (1) aortic smooth muscle protein (pooled from 17-week-old chickens), and (2) osteopontin bone protein extracted from washed (100 % acetone and 100 % trichloroethylene) and ground (with 20 % formic acid) femur bones from 9-week-old chicks. Bone extract was partially purified by filtration (0.8 μm disc filter) and by fractionation *via* a sepharose G-25F column equilibrated with 10 % formic acid. Protein eluates (absorbance read at 280 nm) were pooled and lyophylized. Bone protein was reconstituted in phosphate-buffered saline and used for SDS–PAGE and western blotting (0.5–2.0 μg) as a standard osteopontin protein.

Extraction of total RNA from aortic smooth muscle tissues and neointimal plaques

Birds were quickly decapitated, and the abdominal aorta was removed and placed in chilled aerated Ringer. Neointimal plaques and aortic smooth muscle tissues underlying the plaques or intact aortic smooth muscle tissues were prepared as described above. Tissues from 4–6 birds (*N*) were pooled for one determination (*n*). Tissues were snap-frozen in liquid nitrogen and placed on dry ice; total RNA was extracted by a modification of the method of Glisin et al. (1974; described by Maniatis et al., 1989). Briefly, the frozen tissues were homogenized (Glas-Col homogenizer: motor speed 40) on ice in 4 mol l^{-1} guanidine thiocyanate containing 1 % beta-mercaptoethanol (Sigma Chemical Co.). Approximately 0.5 % sodium lauryl sarcosinate was added to the homogenate, and the homogenate was centrifuged at 6000 revs min^{-1} (4300 ***g***) at ambient temperature. The supernatant was then layered onto a 5.7 mol l^{-1} cesium chloride/10 mmol l^{-1} EDTA (pH 7.5) cushion (1 ml of homogenate per 9 ml of CsCl/EDTA solution) and ultracentrifuged in an SW-41 rotor at 34 000 revs min^{-1} for 22 h at 18 °C (approximately 150 000 ***g*** in a Beckman L8-70M ultracentrifuge; Palo Alto, CA, USA). The total RNA pellet was dissolved in 20 μl of diethyl pyrocarbonate (DEPC)-treated water. The quality of total RNA was examined by running approximately 1.0 μg on a 1.5 % Seakem GTG agarose/2.2 mol l^{-1} formaldehyde gel.

Oligonucleotide synthesis

The following oligonucleotides were synthesized commercially (Great American Gene Company; Ramona, CA, USA). A pair of degenerate chicken osteopontin (termed COPN) primers, COPN1 sense primer (nucleotides 519–541 of the mRNA) 5′-TGCCAGGAAGCTCATTGAGGATG-3′ and COPN2 antisense primer (nucleotides 937–913 of the mRNA) 5′-GCGTCTACATTTACAAACACACGTC-3′, were constructed on the basis of the published nucleotide sequence of chicken osteopontin cDNA from plasmid MMPP2 (a gift from Dr L. C. Gerstenfeld) (Moore et al., 1991). Glyceraldehyde-3-phosphate dehydrogenase primers, GAPDH1 sense primer (nucleotides 198–220 of the mRNA) 5′-ACTTGTGATCAATGGGCACGCCATC-3′ and GAPDH2 antisense primer (nucleotides 679–656 of the mRNA) 5′-CTTCCCATTCAGCACAGGGATGAC-3′ (Milner et al., 1983), and chicken alpha smooth muscle actin primers, AA1 sense primer (nucleotides 5118–5141 of the gene) 5′-GACAGCTATGTAGGTGATGAGGCT-3′ and AA2 antisense primer (nucleotides 6534–6514 of the gene) 5′-AGCCAGGTCCAGACGCATGAT-3′ (Carroll et al., 1986), were also synthesized. The expected lengths of reverse transcription–polymerase chain reaction (RT–PCR) products for GAPDH, chicken osteopontin and chicken alpha smooth muscle actin are, respectively, 481, 419 and 393 base pairs (bp).

Reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA extracted as above was processed for RT–PCR in accordance with reported procedures (Siebert and Larrick, 1992; Araki et al., 1993) with slight modifications. To generate the first-strand cDNA, extracted RNA (0.5–10.0 μg) was reverse-transcribed (total incubation mixture, 20 μl) at 42 °C for 50 min in first-strand buffer (50 mmol l^{-1} Tris, 75 mmol l^{-1}

KCl, 3.0 mmol l^{-1} $MgCl_2$, pH 8.4), 10 mmol l^{-1} dithiothreitol, 0.5 mmol l^{-1} of each dNTP, random primers (3 μg μl^{-1}, 1.5 μl) (Life Technologies) and Superscript II reverse transcriptase (40 units ml^{-1}, Life Technologies). First-strand cDNA (template dilution 1:100) was used in PCR amplification reactions (total incubation mixture, 100 μl) in a reaction buffer containing 20 mmol l^{-1} Tris (pH 8.4), 50 mmol l^{-1} KCl and 1.67 mmol l^{-1} $MgCl_2$, with the specific primer set described above (COPN1, 4.3 pmol l^{-1}; COPN2, 4.1 pmol l^{-1}), *Taq* DNA polymerase (2.5 units, Life Technologies) and 0.2 mmol l^{-1} of each dNTP. Primers for GAPDH (GAPDH1, 3.5 pmol l^{-1}; GAPDH2, 2.7 pmol l^{-1}) and alpha smooth muscle actin (AA1, 3.54 pmol l^{-1}; AA2, 3.86 pmol l^{-1}) were used for some protocols.

PCR amplifications were carried out in a thermocycler (Ericomp, Inc.; Twinblock System Easycycler Series; San Diego, CA, USA) under the following conditions: denaturation at 94 °C for 3 min for one cycle, 35 cycles of denaturation at 94 °C for 45 s each, annealing at 62 °C for 30 s, extension at 72 °C for 45 s, and a final extension for one cycle at 72 °C for 10 min. *In-vitro*-transcribed osteopontin mRNA from plasmid MMPP2 was used as a positive control in all RT–PCR assays. The PCR products were loaded onto agarose gel (3 % Seakem and 1 % NuSieve), electrophoresed and stained with 0.5 μg ml^{-1} ethidium bromide.

Density and statistical analyses

The densities of protein bands from immunoblot analysis and cDNA bands derived from RT–PCR were measured using the NIH Image 1.57 program. Relative densities of osteopontin protein fragments were calculated with respect to the density of partially purified 66 kDa standard bone protein. The density of alpha-smooth muscle actin protein was calculated using the NIH Image program (in arbitrary units) or expressed relative to the density of pooled vascular smooth muscle protein (interassay standard). A two-factor analysis of variance (ANOVA) followed by, when applicable, the Newman–Keuls multiple-comparison test or the least significance difference (LSD) test was used for statistical evaluation.

Results

Osteopontin protein expression

The yield of total protein (μg) per milligram of intact aortic smooth muscle (n=29 determinations; N=50 birds; 6–25 weeks of age, body mass 824±47 g), neointimal plaques (n=9, N=22, 15–45 weeks of age, body mass 1613±41 g) and aortic smooth muscle tissues underlying neointimal plaques (n=12, N=22, 9–45 weeks of age, body mass 1582±45 g) are summarized in Table 1 (values are means ± S.E.M.). There were no significant differences among protein yields from spontaneously developed neointimal plaques, intact aortic smooth muscle or aortic smooth muscle underlying neointimal plaques.

Immunoblot analysis of intact aortic smooth muscle tissues from chicks (6 weeks of age, N=5, pooled; body mass 92±19 g) indicated that rabbit anti-fowl osteopontin antibody recognized proteins of approximately 66–70, 50 and 43 kDa; anti-osteopontin proteins of smaller sizes were more clearly seen in aortic smooth muscle tissues (Fig. 1). In contrast, the immunoblots of proteins extracted from spontaneously developed neointimal plaques (38 weeks of age, N=3, pooled; body mass 1780±59 g) exhibited a major protein band of approximately 66–70 kDa, equivalent to the molecular mass of reported bone osteopontin phosphoprotein, whereas aortic smooth muscle control (interassay standard) expressed three sizes of osteopontin protein (Fig. 2) similar to those shown in Fig. 1. The density of the approximately 66–70 kDa band was higher in neointimal plaques than in aortic smooth muscle tissues, when compared at the same amount of protein (40 μg) loaded onto the gel (Fig. 2).

The relative densities (normalized to values for bone osteopontin protein simultaneously determined in each immunoblot) of various sizes of osteopontin protein bands expressed in neointimal plaques (Fig. 3A) and in aortic smooth muscle tissues (Fig. 3B) were plotted against the amount of protein loaded onto the gel. All sizes of osteopontin proteins show load-dependent increases in density. The load-dependent increase of approximately 66–70 kDa protein bands in neointimal plaques (15–38 weeks of age, N=16, n=5, body mass 1622±46 g) was significantly higher ($0.01<P<0.05$; ANOVA) than that of smaller proteins (Fig. 3A). In contrast, underlying smooth muscle tissues (24–45 weeks of age, N=18, n=6, body mass 1663±24 g, 14 of 18 tissues paired with neointimal plaques) expressed the three sizes of osteopontin proteins in approximately equal amount (Fig. 3B). Furthermore, the approximately 66–70 kDa osteopontin protein expression in neointimal plaques tended to be higher than that in aortic smooth muscle tissues (at 30 μg and 40 μg total RNA loading, $0.01<P<0.05$; LSD). The total amounts of all sizes of osteopontin proteins

Table 1. *Yield of total protein or total RNA from aortic tissues*

Tissue	n	N	Total protein (μg mg^{-1} tissue)	n	N	Total RNA (μg mg^{-1} tissue)
Aortic smooth muscle (ASM)	29	50	0.024±0.002			–
Neointimal plaques	9	22	0.039±0.019	6	20	0.181±0.049
ASM under plaque	12	22	0.028±0.005	10	20	0.147±0.043

n, number of determinations; N, number of birds.
Values are means ± S.E.M.

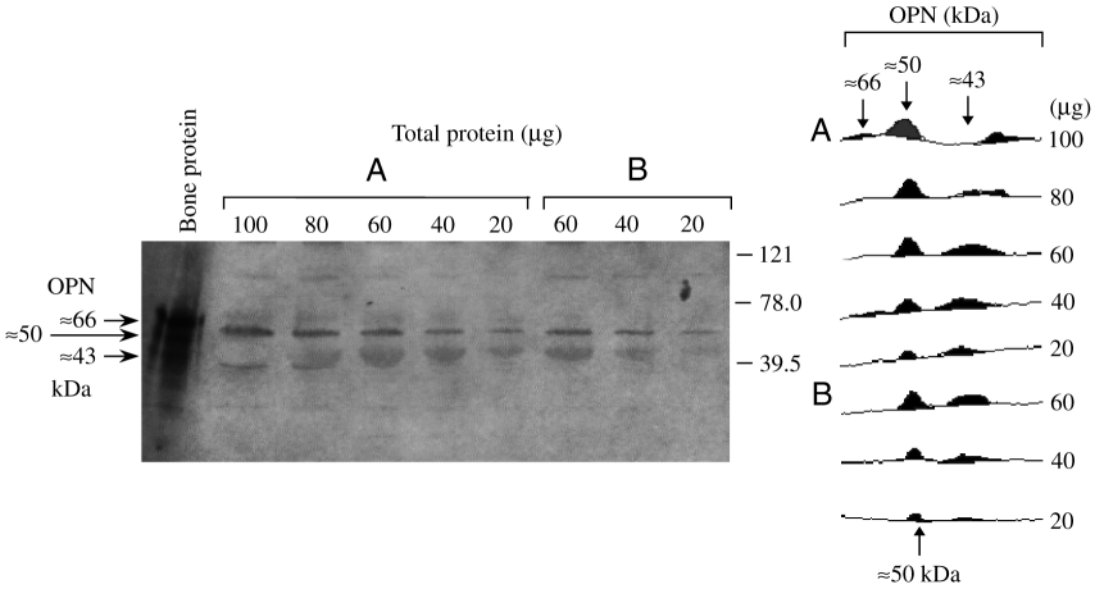

Fig. 1. Osteopontin (OPN) protein detected by immunoblot analysis in intact aortic smooth muscle from 6-week-old chicks (*N*=5 birds, pooled). To examine increases in osteopontin protein levels at different protein loads, various amounts of crude protein extract were electrophoresed on SDS–polyacrylamide gel, and protein bands were detected with rabbit anti-fowl osteopontin antibody (1:750 dilution) and goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad alkaline phosphatase conjugate substrate kit). A and B are two separate determinations from pooled extract. Bone osteopontin (10 μg), extracted and partially purified from chicken femur bones, was used as a standard protein. The right-hand column indicates the density of the protein fragments measured using the NIH Image 1.57 program.

were similar in neointima and underlying aortic smooth muscle.

Alpha smooth muscle actin protein expression

In the present study, we used alpha smooth muscle actin protein as a marker for differentiated vascular smooth muscle cell protein. Immunoblot analysis of 24-week-old young adult hens (*N*=4, body mass 1589±47 g) detected the approximately 43 kDa alpha smooth muscle actin protein band in both neointimal plaques (Fig. 4A) and aortic smooth muscle tissues underlying the plaques (Fig. 4B). In both neointimal plaques and aortic smooth muscle tissues, the densities of protein bands (in arbitrary units) increased as a function of the amount of protein extract loaded (Fig. 5A). Alpha smooth muscle actin protein expression was higher in aortic smooth muscle tissues (24–95 weeks of age, *N*=16, *n*=6, body mass 1638±31 g) than in spontaneously developed neointimal plaques collected from the same group of birds (Fig. 5A) ($P<0.05$; ANOVA). In another set of birds (25–95 weeks of age, body mass 1705±35 g; *N*=14, *n*=5), we compared alpha smooth muscle actin expression in neointimal plaques and underlying aortic smooth muscle at lower loads of protein extract. Densities of alpha smooth muscle actin protein bands were normalized against the densities of pooled aortic smooth muscle protein bands (interassay standard) simultaneously determined in each assay. Alpha smooth muscle actin expression was higher in

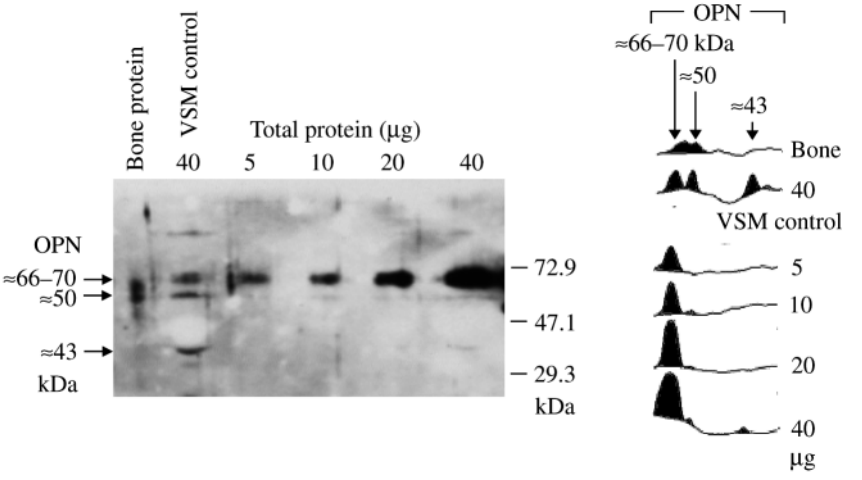

Fig. 2. Osteopontin (OPN) protein in spontaneously developed neointimal plaques (38-week-old, *N*=3 birds, pooled) detected by immunoblot analysis. To examine the effects of increasing amounts of total protein on osteopontin protein expression, various amounts of crude extract were electrophoresed on SDS–polyacrylamide gel, and protein bands were detected with rabbit anti-fowl osteopontin antibody (1:750 dilution) and horseradish-peroxidase-conjugated anti-rabbit IgG (chemiluminescence method). Bone osteopontin (0.5 μg), extracted and partially purified from chicken femur bones, was used as a standard protein, and pooled aortic smooth muscle protein (40 μg) was used for interassay control (VSM control). The right-hand column indicates the density of the protein fragments measured using the NIH Image 1.57 program.

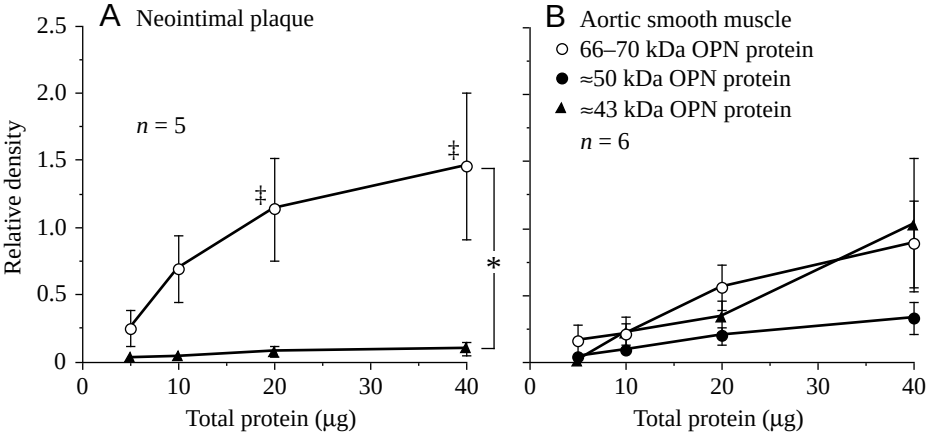

Fig. 3. The relative densities of osteopontin (OPN) proteins (calculated with respect to the amount of 66 kDa bone osteopontin protein in each assay) plotted against the amounts of total protein loaded onto SDS–PAGE gels. (A) Osteopontin protein in neointimal plaques (15–38 weeks of age, N=16 birds, n=5 determinations); (B) osteopontin protein from underlying aortic smooth muscles (24–45 weeks of age, N=18 birds, n=6 determinations). Paired tissues (neointimal plaque and underlying smooth muscle tissues) were collected from 14 of 18 birds. Values are means ± S.E.M. An asterisk indicates a significant difference ($0.01<P<0.05$) in load-dependence between approximately 66–70 kDa osteopontin protein and approximately 43 kDa osteopontin protein (ANOVA). A double dagger indicates a significant difference ($P<0.01$) by Newman–Keuls multiple-comparison test at a given load.

intact aortic smooth muscle than in neointimal plaque when 4 μg and 8 μg were loaded (Fig. 5B) ($P<0.01$; Newman–Keuls multiple-comparison test).

Reverse transcription polymerase chain reaction (RT–PCR)

Table 1 summarizes the yields of total RNA collected from aortic smooth muscle (9–79 weeks of age, N=20, n=6–8) (paired collection) and neointimal plaques. The yield of total RNA from neointimal plaques was similar to that from the underlying aortic smooth muscle tissues (Table 1). Fig. 6 shows the RT–PCR of the total RNA extracted from intact aortic smooth muscle of 12-week-old chickens (N=6, body mass 1082±45 g). The RT–PCR of the total RNA from spontaneously developed neointimal plaques (31-week-old chickens, N=5, body mass 1655±78 g) is shown in Fig. 7. The RT–PCR products of total RNA from aortic smooth muscle (Fig. 6) and neointimal plaques (Fig. 7) both revealed the 481 bp, 419 bp and 393 bp cDNA bands designated, respectively, GAPDH, osteopontin and alpha smooth muscle actin, indicating that mRNA of these molecules is present in both tissues.

Discussion

In domestic fowl, vascular subendothelial hyperplasia and neointimal plaques are first seen in the abdominal aorta slightly above the ischiadic bifurcation (most frequently on the ventral side) and, later, in the branching points of the aorta (Qin and Nishimura, 1998). We found that similar neointimal plaques can be induced by balloon-catheter endothelium injury in chickens (Madison and Nishimura, 1994; Shimada et al., 1998). Both spontaneously developed and endothelium-injury-induced neointimal plaques contain endothelial cells that are often deformed and show signs of degeneration and subendothelial neointimal cells containing abundant endoplasmic reticulum, Golgi apparatus, vesicle structures and abundant extracellular matrix showing the features of the 'synthetic state'. Neointimal cells are thought to be modulated vascular smooth muscle cells that migrate from the media tunica upon stimulation and proliferate in the subendothelial regions (Schwartz et al., 1995). Hence, fowl aortae provide a unique model for comparing biochemical and functional

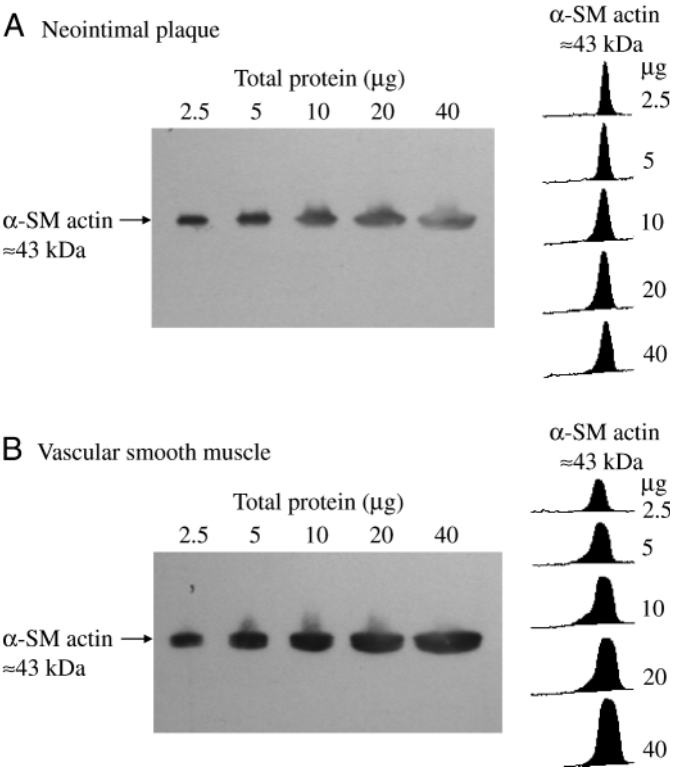

Fig. 4. A representative example of alpha smooth muscle (α-SM) actin protein expression (24-week-old chickens, N=4 birds, pooled) in neointimal plaques (A) and vascular smooth muscle underlying plaques (paired collection) (B). Total protein (2.5–40 μg) was loaded onto 12 % SDS–PAGE gels, blotted and detected by chemiluminescence as described in Materials and methods. The right-hand column indicates the density of the protein fragments measured using the NIH Image 1.57 program.

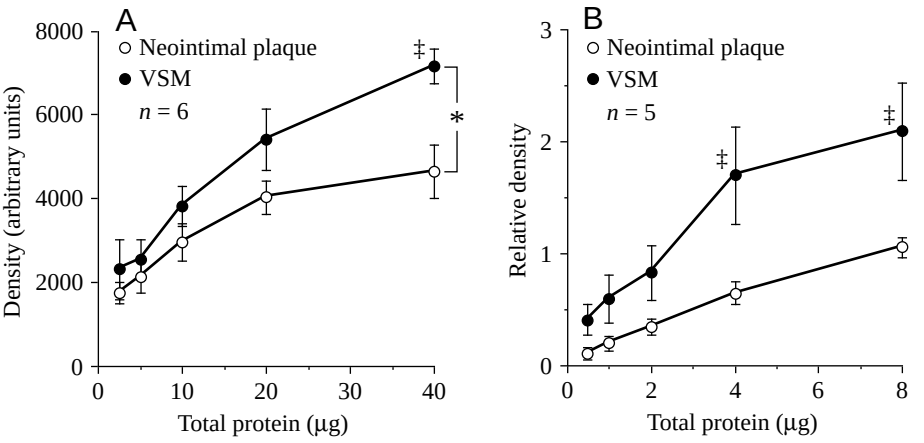

Fig. 5. The densities (A, in arbitrary units; B, relative to the density of the pooled smooth muscle protein interassay standard) of alpha smooth muscle actin protein in neointimal plaques (open circles) and underlying aortic smooth muscle (VSM; filled circles) plotted against the total amount of protein loaded onto SDS–PAGE gels. A (n=6 determinations, N=16 birds) shows a wider range of protein doses, whereas smaller doses are shown in B (n=5 determinations, N=14 birds). Values are means ± S.E.M. An asterisk indicates a significant difference ($0.01<P<0.05$) in load-dependence between neointimal plaques and aortic smooth muscle tissues (ANOVA). A double dagger indicates a significant difference ($P<0.01$) by Newman–Keuls multiple-comparison test at a given load.

phenotypes of spontaneously developed and endothelium-injury-induced neointimal plaques.

Osteopontin is a bone matrix protein synthesized by osteoblasts and contains an adhesive motif, Arg-Gly-Asp (RGD), that binds to cell surface receptor integrins (osteopontin receptor); osteopontin specifically serves as a ligand for the $\alpha_v\beta_3$ integrin (for reviews, see Butler, 1989; Denhardt and Guo, 1993; Horton et al., 1995). These phosphorylated extracellular matrix proteins appear to play an important role in the bone mineralization process. Osteopontin has also been found in a variety of tissues, including brain, bone marrow, many epithelial cell surfaces, tumor cells, kidney, placenta, inner ear and decidua (Butler, 1989). Osteopontin mRNA and protein are present in intact vascular smooth muscle; osteopontin protein is overexpressed in rat arteries after balloon-catheter-induced endothelium injury (Giachelli et al., 1993; Yamamoto et al., 1997), in human atheromatous plaques (Giachelli et al., 1993) and in cultured

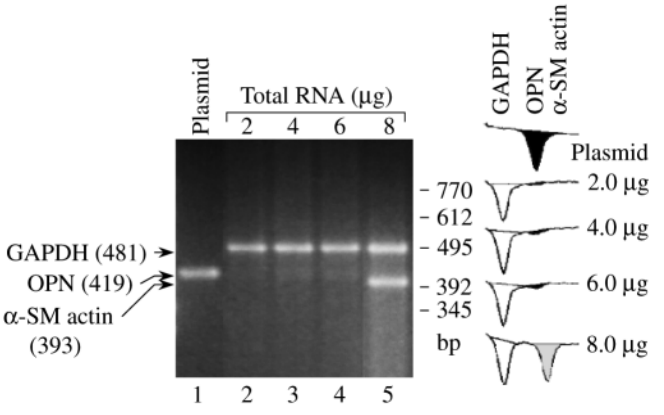

Fig. 6. Reverse transcription–polymerase chain reaction (RT–PCR) products of increasing amounts (2–6 μg) of total RNA extracted from intact vascular smooth muscle (pooled from 12-week-old chickens, N=6 birds). Amplified cDNAs were electrophoresed onto an agarose gel (3% Seakem + 1% NuSieve) and stained with ethidium bromide. The chicken osteopontin (OPN) primers, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers and α-smooth muscle actin (α-SM) primers were added prior to PCR and should produce, respectively, 419 bp, 481 bp and 393 bp products. Lane 1, osteopontin plasmid; lanes 2–4, RT–PCR products from increasing loads of total RNA incubated with osteopontin and GAPDH primers; lane 5, RT–PCR products from 8 μg of RNA incubated with primers for osteopontin, GAPDH and alpha smooth muscle actin. In lane 5, the product of osteopontin primers co-migrates with that of alpha smooth muscle actin. The right-hand column indicates the density of the cDNA bands measured by the NIH Image 1.57 program. See Materials and methods for RT–PCR incubation, primer sequence and concentration. bp, base pairs.

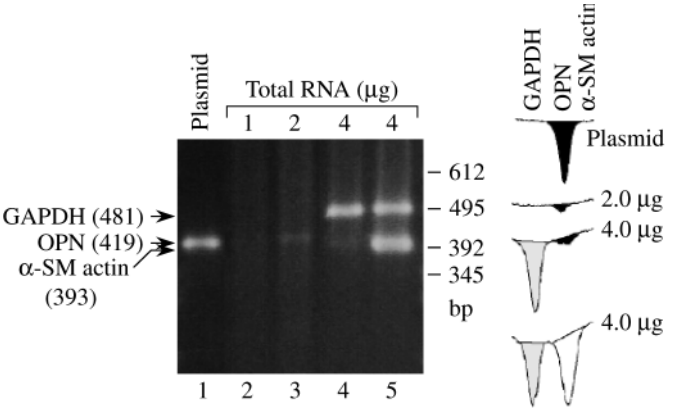

Fig. 7. Reverse transcription–polymerase chain reaction (RT–PCR) products from neointimal plaque total RNA (1.0–2.0 μg) pooled from 31-week-old adult hens (N=5 birds). Amplified cDNAs were electrophoresed onto an agarose gel (3% Seakem + 1% NuSieve) and stained with ethidium bromide. Primers are the same as in Fig. 6. Lane 1, osteopontin (OPN) plasmid; lanes 2 and 3, RT–PCR product of total RNA incubated with osteopontin primers; lane 4, the product after incubation with osteopontin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers; lane 5, the product after incubation with osteopontin, GAPDH and alpha smooth muscle actin primers. In lane 5, the products from osteopontin and alpha smooth muscle actin primers co-migrate. The right-hand column indicates the density of the cDNA bands measured by the NIH Image 1.57 program. bp, base pairs.

aortic smooth muscle cells of the synthetic phenotype (Yamamoto et al., 1997). The approximately 66 kDa chicken bone phosphoprotein and a genomic clone of this phosphoprotein-encoding gene have been isolated from chick embryonic osteoblasts (Gotoh et al., 1990). Fowl osteopontin has a recognition sequence for integrin binding, Arg-Gly-Asp (RGD) (Gerstenfeld et al., 1990; Moore et al., 1991; Rafidi et al., 1994). An approximately 35 % similarity in protein sequence, but a 60 % similarity at the nucleotide level, has been noted between the approximately 66 kDa chicken bone phosphoprotein and the mammalian osteopontin (Moore et al., 1991).

In the present study of chicken aortic smooth muscle tissues and neointimal plaques, we found that rabbit anti-fowl osteopontin antibody recognized a 66–70 kDa protein that migrates like partially purified chicken bone protein. We also noted two additional proteins of smaller sizes (approximately 50 kDa and 43 kDa) in chicken aortic smooth muscle tissues. Spontaneously developed neointimal plaque predominantly contains the 66–70 kDa osteopontin protein, while it contains only traces of smaller osteopontin proteins. In contrast, approximately equal amounts of three osteopontin proteins were present in intact aortic smooth muscle and in the aortic smooth muscle underlying neointimal plaques. The level of the approximately 66–70 kDa osteopontin protein appears to be higher in neointimal plaques than in underlying aortic smooth muscle tissues.

Osteopontin proteins of smaller molecular mass have also been reported in chicken bone (Gerstenfeld et al., 1990). They appear to be osteopontin metabolites produced *in vivo* by proteolytic enzymes during accumulation within the extracellular matrix. Furthermore, low-molecular-mass variants of osteopontin species, possibly generated by serine proteinases, have been noted in the urine of patients with kidney stones (Bautista et al., 1996). The predominance of unmetabolized osteopontin in neointimal plaques suggests that osteopontin protein synthesis takes place with little subsequent processing. In contrast, osteopontin metabolism may be more active in normal aortic smooth muscle tissues. This agrees with the morphological observation that neointimal plaques contain abundant extracellular matrix (H. Nishimura, Z. Xi, H. Kempf, R. F. Wideman and P. Corvol, unpublished observation). Using RT–PCR analysis, we investigated whether osteopontin mRNA is expressed in aortic smooth muscle and neointimal plaques. The total RNA extracted from intact aortic smooth muscle tissues and neointimal plaques exhibited PCR products corresponding to the product from recombinant plasmid containing an osteopontin cDNA, suggesting that osteopontin mRNA is present and that osteopontin is synthesized in both aortic smooth muscle and spontaneously developed neointimal plaques.

Quiescent vascular smooth muscle expresses cytoskeletal proteins, including alpha smooth muscle actin and smooth muscle myosin heavy chains, smooth muscle myosin light chains, vinculin and intermediate filaments (vimentin and desmin) (Owens, 1995). Among these cytoskeletal proteins, smooth muscle actin and myosin appear to be powerful differentiation markers for studying phenotypic modulation during development and in vascular disease (Mori and Saito, 1996). The present study indicates that, in adult fowl, both neointimal plaques and aortic smooth muscle express alpha smooth muscle actin; the level of expression is lower in spontaneously developed neointima than in intact aortic smooth muscle tissues, suggesting that phenotypic modulation from contractile to synthetic phenotypes may have occurred in the neointima. This observation is consistent with the view that the actin profile in mammals changes in atherosclerotic vessels (Campbell and Campbell, 1985; Schwartz et al., 1995). Furthermore, Gabbiani and coworkers (Gabbiani et al., 1984; Babaev et al., 1990; Desmouliere and Gabbiani, 1992) demonstrated that, in rat aortic hyperplasia, human atheromatous plaques and cultured rat aortic cells, there is a typical switch in actin expression with a predominance of the beta-isoform over the alpha-isoform. The presence of smooth muscle actin isoforms in fowl neointima remains to be confirmed.

The role of osteopontin and the physiological implication of osteopontin expression in spontaneously developed neointima in chickens (present study) and endothelium-injury-induced neointima in rats (Giachelli et al., 1993, 1995) are not well understood. Recent studies in cell biology indicate that extracellular matrix proteins such as fibronectin and osteopontin, *via* interaction with cell surface adhesion receptors, particularly integrins, modulate cytoskeletal organization and various cell functions, including growth, differentiation and motility (Schwartz et al., 1995; Schwartz, 1997; Ruoslahati and Engvall, 1997). Migrating cells must generate traction forces at the site of adhesion through integrins that bind simultaneously to the extracellular matrix *via* their extracellular domains and to cytoskeletal elements through their cytoplasmic domains (Choquet et al., 1997; Lauffenburger and Horwitz, 1996). Endothelium-injury-induced diffusible molecules (Powell et al., 1996) may evoke initial mechanical and biochemical signal pathways that lead to assembly of cytoskeleton-associated proteins. This, in turn, leads to integrin binding and other signaling events *via* protein phosphorylation (Schwartzbauer, 1997). Indeed, it has been reported that purified osteopontin stimulates endothelial cell (Liaw et al., 1995) and aortic smooth muscle cell (Yue et al., 1994) adhesion and migration *in vitro via* $\alpha_v\beta_3$ integrin and inhibits nitric oxide production (Rollo et al., 1996), whereas treatment with anti-osteopontin antibody (Liaw et al., 1997) or with the antibody to the RGD-containing peptide that inhibits integrins reduces the size of the neointima and/or cell proliferation (Matsuno et al., 1994; Choi et al., 1994). Preliminary studies indicate that rabbit anti-chicken osteopontin antibody inhibits the migration of cultured fowl aortic smooth muscle cells (Kuykindoll and Nishimura, 1999; H. Nishimura, R. J. Kuykindoll and X. Wenbo, unpublished observations). Furthermore, osteopontin mRNA expression is increased after stimulation of quiescent rat aortic smooth muscle cell cultures with serum (Gadeau et al., 1993),

suggesting that osteopontin expression is associated with cell proliferation. Further studies are needed to determine the role of osteopontin in the development of neointimal lesions following vascular injury or atherosclerosis.

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