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ORIGINAL ARTICLE

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Expression of inducible nitric oxide synthase in macrophages and smooth muscle cells in various types of human atherosclerotic lesions

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Abstract Nitric oxide (NO) is an important regulatory agent in blood vessels. We studied the expression of inducible nitric oxide synthase (iNOS) in different types of human atherosclerotic lesions using simultaneous in situ hybridization and immunocytochemistry. Since nitric oxide and its derivates or reaction products can have both oxidative and antioxidative effects, we also studied the presence of oxidized low-density lipoproteins (ox-LDL) and peroxynitrite-modified proteins in the same lesions as indicators of oxidative damage. Twenty-seven aortic samples were studied from seven autopsies. Samples were classified microscopically as normal areas, initial lesions (type I), fatty streaks (type II), intermediate lesions (type III), atheroma (type IV), fibroatheroma lesions (type Va) and fibrotic lesions (type Vc). In normal arterial wall iNOS mRNA was expressed at a low level in smooth muscle cells (SMCs). Absence of, or a low level of, epitopes characteristic of ox-LDL was found in the normal arterial wall. The expression of iNOS mRNA and protein was induced in macrophages and SMCs in the majority of early lesions and in all advanced atherosclerotic lesions. Epitopes characteristic of ox-LDL and peroxynitrite-modified proteins tended to be colocalized in iNOS-positive lesions. We consider that iNOS and oxidative injuries may play an important part in atherogenesis.

Key words Inducible nitric oxide synthase · Macrophages · Smooth muscle cells · Oxidized LDL · Peroxynitrite · Atherogenesis

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Introduction

Several lines of evidence suggest that nitric oxide (NO) plays an important part in vascular physiology and pathology [1–3]. NO regulates vascular tone, and inhibits SMC proliferation and platelet aggregation [1, 2, 4]. NO can also suppress lipoprotein oxidation [5–8] and inhibit lipid peroxidation by terminating radical chain reactions [9]. NO is synthesized by three types of nitric oxide synthases (NOS; for review see [10]): constitutively expressed neuronal NOS (NOS I) [11], constitutively expressed endothelial NOS (NOS III) [12] and inducible (i)NOS (NOS II) [13]. Endothelial NOS III is responsible for basal production of NO in arteries, whereas iNOS is expressed under inflammatory conditions [1, 2, 4]. iNOS produces much larger quantities of NO than NOS III and may play a part in cellular damage, inflammation and apoptosis.

Many harmful effects of NO are thought to be caused by the reaction of NO with superoxide anion. This reaction occurs very rapidly and produces highly toxic peroxynitrite ($ONOO^-$) which can directly damage lipids and proteins [14]. The production rate of peroxynitrite is dependent on the molar ratio of NO to superoxide anion. The reaction of NO with superoxide anion may be one of the factors involved in the generation of oxidative damage in atherogenesis. Since cellular content, cellular activation and the structure of the artery change dramatically in different types of atherosclerotic lesions [15] it is possible that the expression of iNOS and other pro- and antioxidative factors change during atherogenesis. In the normal arterial wall the amount of NO, produced mainly by endothelial NOS III, is sufficient to maintain an excess of NO. Hypercholesterolaemia, while inducing iNOS expression, also causes increased superoxide anion production and endothelial dysfunction [16]. This may result in a decreased NO-to-superoxide anion ratio [17], which favours the production of peroxynitrite [18]. Therefore, the balance of NO to superoxide anion seems to be an important factor that may have important consequences in arterial wall pathology.

Table 1 Expression of iNOS, and presence of epitopes characteristic of ox-LDL and peroxynitrite-modified proteins in different types of atherosclerotic lesions in human aorta (*M* Male, *F* female. – no detectable expression/immunostaining, + weak expression/immunostaining, ++ moderate expression/immunostaining, +++ strong expression/immunostaining, *NA* not available, *Abd* abdominal, *Thor* thoracic)

Age/sex	Tissue sample	Anatomical site	Lesion type [15]	iNOS	Epitopes characteristic of ox-LDL	Peroxynitrite modified proteins
51/F	1	Abd.	Normal	+	+	+
52/M	2	Abd.	Normal	+	–	–
52/M	2	Abd.	I	++	++	++
51/F	3–6	Thor.	II	+++	+	++
43/M	7	Thor.	II	++	++	+
51/F	8	Abd.	II	++	+++	+
53/M	9–10	Abd.	II	–	++	+++
53/M	11	Thor.	II	–	+	++
51/F	12	Thor.	III	+++	+++	+++
52/M	13–14	Abd.	III	++	++	++
41/M	15	Thor.	III	+	NA	NA
73/M	16–17	Abd.	IV	++	++	++
51/F	18	Thor.	IV	+++	+++	+++
52/M	19–20	Thor.	IV	++	NA	NA
52/M	21–22	Abd.	IV	+++	+++	+++
73/M	23–26	Thor.	Va	+++	+++	+++
52/M	27	Abd.	Vc	++	+++	+++

Although it has been clearly shown that mouse macrophages express iNOS [1, 2, 4], it has been difficult to demonstrate the expression of iNOS in human macrophages or in human atherosclerotic lesions [19–21]. Also, it is important to know which cell types express iNOS at different stages of atherogenesis and whether there is any colocalization between iNOS expression and the presence of oxidized lipoproteins and peroxynitrite-modified proteins in atherosclerotic lesions. The recent classification of atherosclerotic lesions by Stary et al. [15] has clarified definitions of various types of lesions and will allow better comparison of data between different laboratories. This classification was used in the present study.

The purpose of the present study was to analyse different types of human atherosclerotic lesions to find out which cell types express iNOS during the progression of human atherosclerosis. Our study showed that iNOS mRNA and protein are expressed in macrophages and SMCs in the majority of early lesions and in all advanced atherosclerotic lesions. Oxidized lipoproteins and peroxynitrite-modified proteins tended to colocalize with iNOS-positive lesions. The findings suggest an important role for iNOS in the pathogenesis of atherosclerosis.

Methods

Aortic samples were collected from seven autopsies (six men, ages 41, 43, 49, 52, 53 and 73 years; one woman, age 51 years; Table 1). Post-mortem time varied from 4 h to 13 h. Paraffin-embedded and frozen sections were used for the study. Samples were immediately transferred either to formal-sucrose at 4°C (4% paraformaldehyde, 15% sucrose, 1 mmol/l EDTA, 50 µmol/l butylated hydroxytoluene, pH 7.4) [22] or frozen in OCT compound (Miles Laboratories, Elkhart, Ind.). Formal-sucrose samples were fixed for 4 h, rinsed for 20 h in 15% sucrose containing 1 mmol/l EDTA and 50 µmol/l butylated hydroxytoluene and embedded in paraffin. Samples were used for in situ hybridization and immunocytochemical studies as described below. We cannot exclude the possibility of post-mortem changes in the tissue samples. However, no major changes were observed in previous studies conducted on similar tissue samples, compared with results obtained from organ donors or from perfusion-fixed animals [22–24].

Aortic samples were classified microscopically according to Stary et al. [15] as normal areas, type I (initial lesions), type II (fatty streaks; mostly macrophage-rich progressive lesions), type III (intermediate lesions), type IV (atheroma), type Va (fibroatheroma) and type Vc (fibrotic lesion) lesions. Twenty-seven aortic samples of different types of lesions were selected for the study (Table 1). Even though the age range of the studied cases was quite narrow, we think the results are representative since both early and late lesions can be found in the same individuals and lesion characteristics are usually related to the type of the lesion and not to the age of the patient [15]. All human studies were approved by the Ethical Committee of the University Hospital of Kuopio.

An antisense mouse iNOS riboprobe (nucleotides 201–1017) [25] was used for in situ hybridization studies. Homology between the mouse iNOS probe and the corresponding human iNOS sequence is 78%. iNOS sense riboprobe was used as a control [25]. For riboprobe synthesis, the iNOS cDNA was subcloned in pBluescript plasmid (Stratagene, La Jolla, Calif.) using standard techniques [26]. Antisense and sense riboprobes were synthesized using T3- or T7-RNA polymerases with ^{35}S-UTP (1000–1500 Ci/mmol, New England Nuclear, Boston, Mass.) as described elsewhere [22]. All reagents used for riboprobe synthesis were supplied by Promega (Madison, Wisc.).

Serial sections were used for in situ hybridization studies as described [22, 23]. Briefly, tissue sections were deparaffinized, rehydrated, treated with proteinase K, acetylated, dehydrated and dried in vacuo. Frozen sections were thawed, fixed with formal-sucrose for 10 min, dehydrated and dried in vacuo, after which 50 µl of hybridization solution containing 6×10^6 cpm/ml of each labelled probe was added to tissue sections and the sections were hybridized at 52°C for 14 h. Hybridization solution contains 50% formamide (Fluka Chemie, Buchs, Switzerland), 2×SSC, 20 mmol/l Tris (pH 7.4), 1×Denhardt's solution, 1 mmol/l EDTA, 10% dextran sulphate (Pharmacia Biotech, Uppsala, Sweden), 1 mmol/l dithiothreitol and 0.5 mg/ml yeast tRNA (Boehringer Mannheim, Biochemicals, Germany). 1×SSC contains 150 mmol/l NaCl, 15 mmol/l Na-citrate, pH 7.0. Denhardt's solution contains 0.02% Ficoll and 0.02% BSA (all from Sigma Chemical Co., St. Louis, Mo.). After hybridization the sections were washed three times (once for 30 min. and twice for 5 min) in 4×SSC at 37°C. The sections were then washed at 37°C in 2×SSC and 1×SSC (15 min each). The final wash was at 55°C in 0.1×SSC for 30 min. Tissue sections were dehydrated, dried, dipped in autoradiographic emulsion (NTB-2, Eastman-Kodak, Rochester, N.Y.), and developed after 2–10 weeks' exposure time. After development the sections were counterstained with haematoxylin-eosin. Nonhybridiz-

ing sense riboprobe was used as a control. For some antibodies immunocytochemistry was performed on the same sections before dipping into the autoradiographic emulsion.

Immunostainings were done in serial paraffin and frozen sections [22] using the following antibodies: mouse mAb against human macrophages (HAM-56, DAKO, Glostrup, Denmark) [27], mouse mAb against smooth muscle cells (HHF-35, Enzo Diagnostics, Farmingdale, N.Y.) [28], mouse mAb against iNOS (clone 6, Transduction Laboratories, Exeter, UK) [25], guinea pig polyclonal antisera against malondialdehyde (MDA)-modified LDL (MAL-2) [29], and rabbit polyclonal antiserum against nitrotyrosine residues [30]. Avidin–biotin–horseradish peroxidase system with diaminobenzidine tetrahydrochloride as a chromogen (Vector Laboratories, Burlingame, Calif.) was used for the signal detection. The following controls were used for the immunostainings: incubations with primary antibodies replaced by irrelevant class- and species-matched antibodies and incubations with primary antibodies omitted [22, 23]. The specificity of nitrotyrosine immunostaining was confirmed by blocking the staining with 10 mmol/l 3-nitrotyrosine [30]. Semiquantitative microscopical evaluation of the sections was done by one experienced pathologist (J.S.L.) in random order without knowledge of the origin of the samples. Studied lesions were graded using the following criteria: no detectable expression/immunostaining; weak expression/immunostaining: less than 10% of the lesion cells/area was positive for the studied signal; moderate expression/immunostaining, meaning that 10–50% of the lesion cells/area was positive for the studied signal; strong expression/immunostaining, meaning that more than 50% of the lesion cells/area was positive for the studied signal.

Micrographs were taken by digital camera (SenSys KAF1400-G2, Photometrics, Tucson, Ariz.), processed with digital image processing software (Image-Pro Plus, Media Cybernetics, Silver Spring, Md.) and printed using a sublimation printer (Kodak DS 8650, Eastman-Kodak, Rochester, N.Y.).

Results

iNOS mRNA and protein expression was studied in 27 human aortic samples representing normal aortic intima and atherosclerotic lesions types I–V. Lesions were classified according to Stary et al. [15]. Table 1 summarizes the expression of iNOS mRNA and epitopes characteristic of ox-LDL and peroxynitrite modified proteins in various types of lesions. In normal aorta iNOS mRNA was expressed at low level in SMCs (Fig. 1A). Absence of or a low level of epitopes characteristic of ox-LDL was found in the normal arterial wall (Fig. 1B). In type-I lesions simultaneous in situ hybridization and immunostaining typically showed a presence of macrophages in the subendothelial space and confirmed the expression of iNOS mRNA in lesion macrophages (Fig. 1C). Similar macrophage-containing areas stained positive for iNOS protein (data not shown), epitopes characteristic of ox-LDL (data not shown) and nitrotyrosine residues indicative of the presence of peroxynitrite-modified proteins (Fig. 1D). Figures 1E and 1F show an example of a type-II lesion consisting of layers of macrophages and SMCs. In situ hybridization with iNOS antisense riboprobe immunostained simultaneously against macrophages revealed the expression of iNOS mRNA in lesion macrophages (Fig. 1E). Similar studies with immunostaining against SMCs showed the expression of iNOS mRNA in lesion SMCs (Fig. 1F). However, three of the nine type-II lesions studied were negative for iNOS expression, but all were positive for epitopes characteristic of ox-LDL and peroxynitrite-modified proteins (Table 1).

Aortic samples from a type-III lesion, which represents a transition stage between type-II and type-IV lesions, are shown in Fig. 2. Simultaneous in situ hybridization with iNOS antisense riboprobe and immunostaining against SMCs showed strong iNOS mRNA expression in type-III lesions in the subendothelial area and in lesion SMCs (Fig. 2A, B). Figure 2C shows the expression of iNOS mRNA in macrophages. Immunostainings of serial sections against iNOS protein (data not shown), nitrotyrosine residues (Fig. 2E) and epitopes characteristic of ox-LDL (Fig. 2F, G) showed that iNOS mRNA, peroxynitrite-modified proteins and ox-LDL

Fig. 1A–F iNOS mRNA and protein expression and oxidatively modified epitopes in normal human aorta, and in representative type-I (initial lesion) and type-II (fatty streak) atherosclerotic lesions. **A**, **B** An example of normal abdominal aorta of a 52-year-old man. **A** In situ hybridization with ^{35}S-UTP-labelled iNOS antisense riboprobe immunostained simultaneously against macrophages (HAM-56; dilution 1:500) showing the expression of iNOS mRNA as *black spots* (bright field illumination). No macrophages are present in normal arterial wall. *Arrows* indicate SMC positive for iNOS mRNA. **B** A serial section immunostained against epitopes characteristic of ox-LDL (MAL-2; dilution 1:500). No ox-LDL is present in normal arterial wall. **C**, **D** Sections showing an example of a type-I lesion from abdominal aorta of a 52-year-old man. **C** In situ hybridization with ^{35}S-UTP-labelled iNOS antisense riboprobe. The section was simultaneously immunostained against macrophages (HAM-56; dilution 1:500). iNOS mRNA is shown as *black spots* and macrophages in *brown*. An *arrow* indicates a macrophage positive for iNOS mRNA. **D** Immunostaining of a serial section against nitrotyrosine residues (rabbit antiserum, dilution 1:250) showing the presence of peroxynitrite-modified proteins in the same area expressing iNOS mRNA. An *arrow* indicates positive staining for nitrotyrosine residues. **E**, **F** Sections showing an example of a type-II lesion from the abdominal aorta of a 51-year-old woman. **E** In situ hybridization with iNOS antisense riboprobe immunostained simultaneously with an antibody against macrophages (HAM-56, dilution 1:500). *Arrows* indicate macrophages that express iNOS mRNA. **F** A serial section hybridized with iNOS antisense riboprobe and immunostained simultaneously against SMC (HHF-35, dilution 1:200). *Arrows* indicate SMC that express iNOS mRNA. Control incubations with class- and species-matched immunoglobulins and sense riboprobes were negative (data not shown). An *asterisk* indicates the location of internal elastic lamina. Haematoxylin-eosin counterstaining, *bars* 50 μm

Fig. 4A–F iNOS mRNA and protein expression and oxidatively modified epitopes in serial sections of a representative type Va (fibroatheroma) human atherosclerotic lesion from a 73-year-old man. **A** In situ hybridization with ^{35}S-UTP-labelled iNOS antisense riboprobe showing strong expression of iNOS mRNA in the upper part of the lesion (polarized light epiluminescence image). *Brackets* in **A–F** show the same area. **B** Serial section immunostained for macrophages (HAM-56, dilution 1:500). **C** Serial section immunostained for SMC (HHF-35, dilution 1:100). **D** Serial section immunostained for MDA-lysine epitopes characteristic of ox-LDL (MAL-2, dilution 1:1000). *Arrows* indicate positive staining. **E** Serial section immunostained against nitrotyrosine residues (rabbit antiserum, dilution 1:250). *Arrows* indicate positive staining for peroxynitrite-modified proteins. **F** Nonimmune control for immunostainings (first antibody omitted). Control in situ hybridization with sense riboprobe was negative (data not shown; *c* lesion core). Haematoxylin-eosin counter staining in all sections, *bars* 50 μm

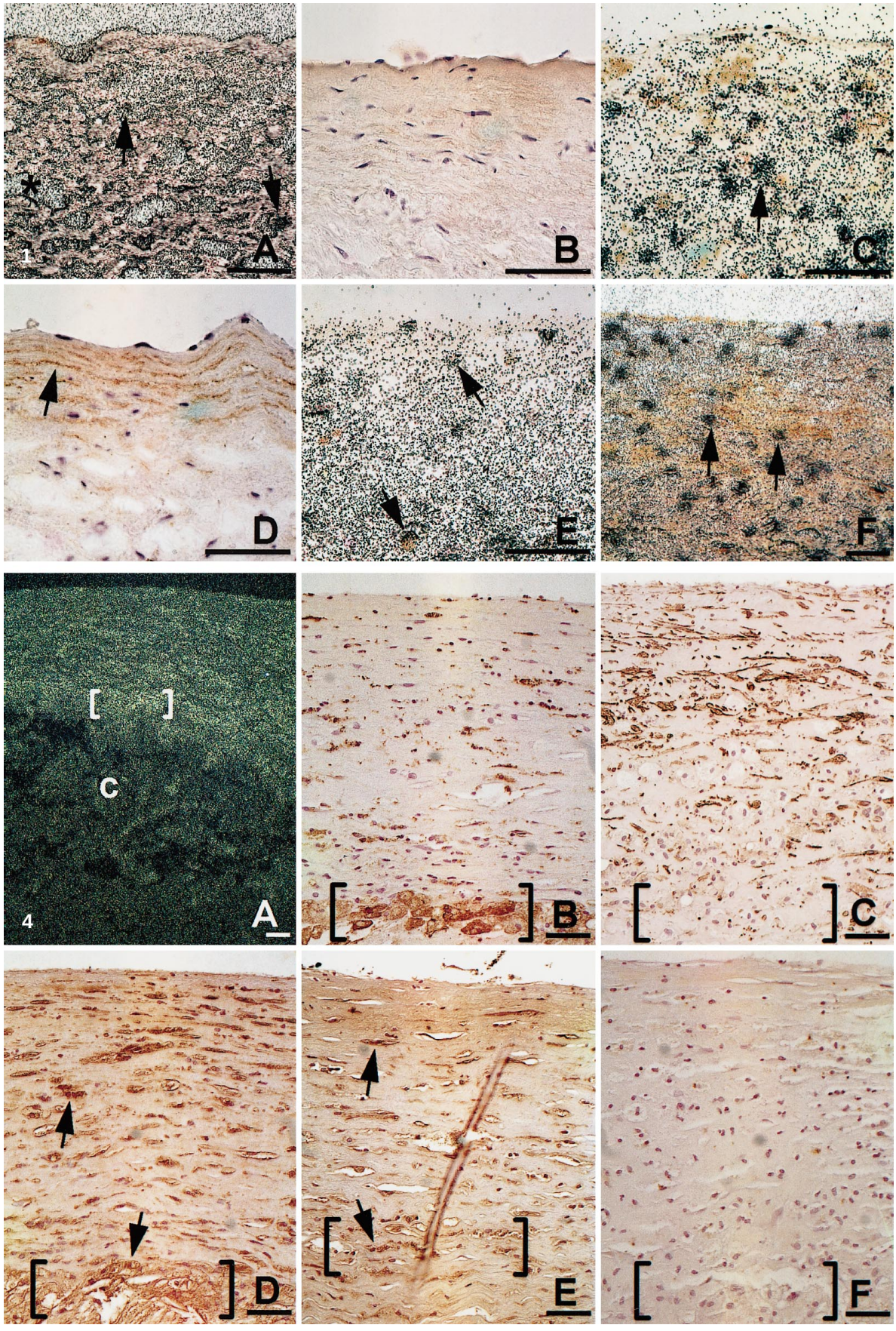

Fig. 1A–F, Fig. 4A–F

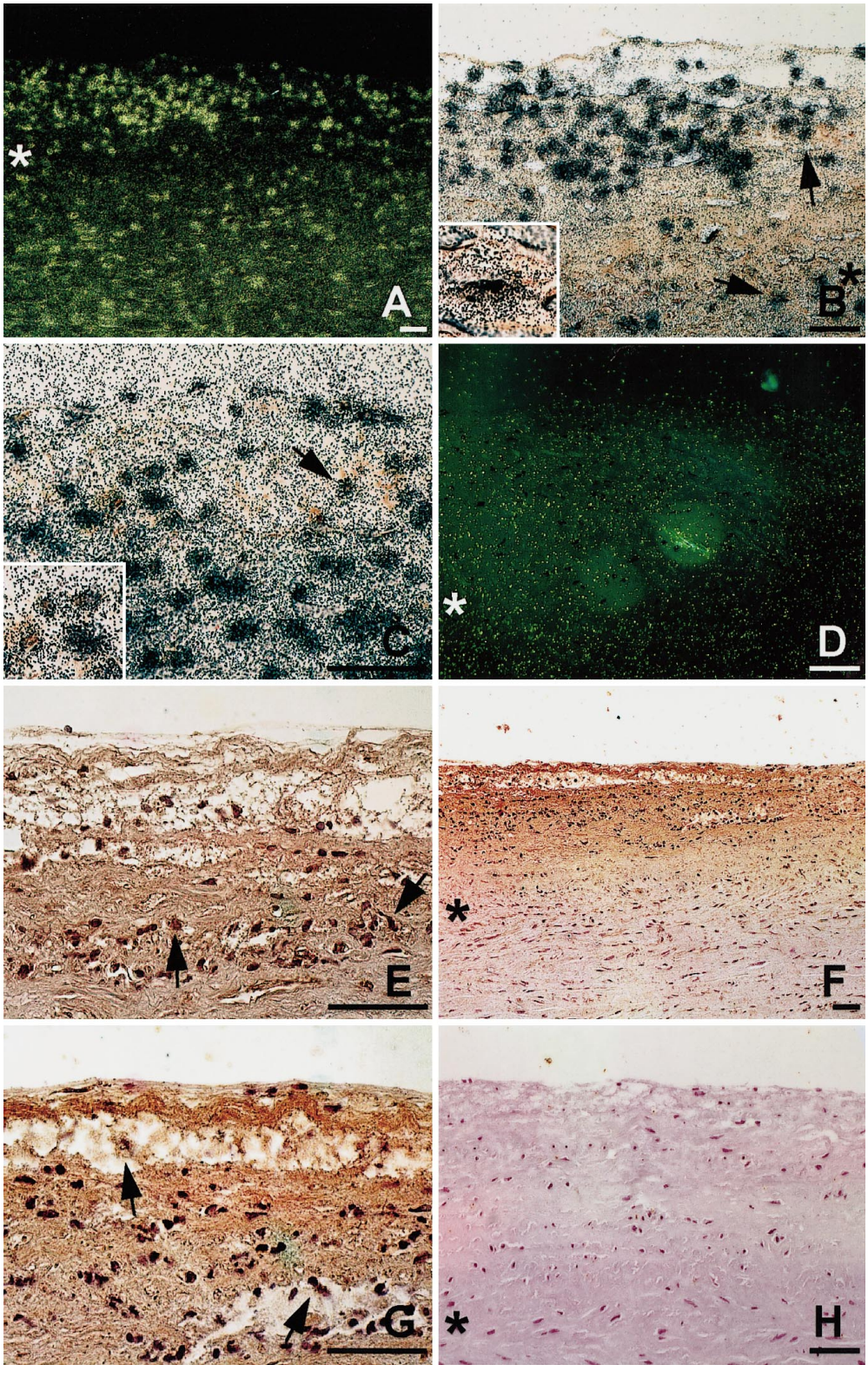

Fig. 2A–H iNOS mRNA and protein and oxidatively modified epitopes in a representative type-III (intermediate) human atherosclerotic lesion from a 51-year-old woman's thoracic aorta. **A** Dark field image of a section hybridized with ^{35}S-UTP-labelled iNOS antisense riboprobe and immunostained simultaneously against SMC (HHF-35, dilution 1:200). iNOS mRNA expression is shown as *bright spots*. **B** Same section as in **A** under bright field illumination. *Arrows* indicate iNOS expression in SMC. *Insert*: higher magnification. **C** Serial section hybridized with iNOS antisense riboprobe and immunostained simultaneously against macrophages (HAM-56, dilution 1:500). *Arrow* indicates a macrophage expressing iNOS mRNA. Insert: higher magnification. **D** In situ hybridization control hybridized with iNOS sense riboprobe. **E** A serial section immunostained against peroxynitrite-modified proteins (rabbit antiserum, dilution 1:250). *Arrows* indicate immunostaining positive for nitrotyrosine residues. **F** Serial section stained against epitopes characteristic of ox-LDL (MAL-2, dilution 1:1000). **G** Same section as in **F**, but at higher magnification. *Arrows* indicate positive staining. **H** Nonimmune control for the immunostainings (first antibody omitted). **A** and **D** are polarized light epiluminescence images. An *asterisk* indicates the location of internal elastic lamina. Haematoxylin-eosin counterstaining, *bars* 50 µm

tended to colocalize in the same areas. Controls for in situ hybridization (Fig. 2D) and for immunocytochemistry (Fig. 2H) were negative.

A type-IV lesion consists of a large, disruptive core of extracellular lipid. A type-V lesion consists of a prominent area of new fibrous connective tissue that may also contain lipid and calcification. Figures 3 and 4 show typical examples of human type-IV and type-V atherosclerotic lesions, respectively. In situ hybridization studies of a type-IV lesion showed clear iNOS mRNA expression

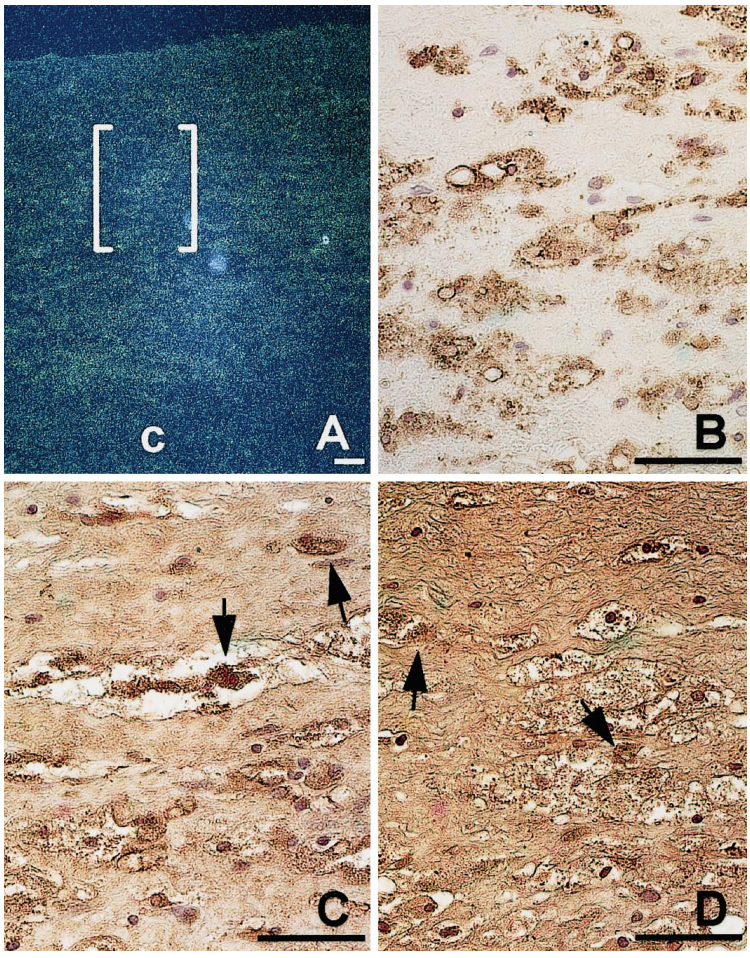

Fig. 3A–D iNOS mRNA and protein expression and oxidatively modified epitopes in serial sections of a representative type IV (atheroma) human atherosclerotic lesion from the aorta of a 73-year-old man. **A** In situ hybridization with ^{35}S-UTP-labelled iNOS antisense riboprobe, showing the expression of mRNA as *bright spots* (polarized light epiluminescence image). *Bracketed area* is shown in **B–D**. **B** Serial section immunostained against macrophages (HAM-56; dilution 1:500). **C** Serial section immunostained against epitopes characteristic of ox-LDL (MAL-2; 1:1000). *Arrows* indicate positive staining for MDA-lysine epitopes. **D** Immunostaining for iNOS protein (clone 6, dilution 1:10). *Arrows* indicate positive staining for iNOS protein. Control incubations with class- and species-matched immunoglobulins and sense riboprobes were negative (data not shown; *c* lesion core). Haematoxylin-eosin counterstaining, *bars* 50 µm

in macrophage-rich areas (Fig. 3A, B), whereas less prominent expression of iNOS mRNA was found in the deeper parts of the lesion (marked "c" in Fig. 3A). Immunostainings of serial sections for macrophages (Fig. 3B), epitopes characteristic of ox-LDL (Fig. 3C) and iNOS protein (Fig. 3D) showed that iNOS mRNA, protein and ox-LDL tended to colocalize in the same areas. SMCs also expressed iNOS in type IV lesions (data not shown).

In situ hybridization of type-V lesions showed the expression of iNOS mRNA in the upper part of the lesion, whereas the expression of iNOS was weaker in the deeper parts of the lesion (Fig. 4A). Immunocytochemistry studies of serial sections showed that iNOS mRNA was expressed in both macrophages and SMCs (Fig. 4B, C). The same area contained epitopes characteristic of ox-LDL and nitrotyrosine residues (Fig. 4D, E). Cellular contents in type V lesions were usually decreased. However, studies on the expression of iNOS mRNA and protein showed no major differences from lesions of types III–IV, except for a tendency for a shift in the iNOS expression towards the upper part of the lesion.

Discussion

NO produced by constitutively expressed endothelial NOS III [31] maintains homeostasis, regulates vasorelaxation and inhibits SMC proliferation and platelet aggregation [1, 2, 4]. iNOS, which produces much higher quantities of NO than NOS III, is expressed at low levels in normal aortic SMCs. As shown in the present study, iNOS expression is induced in macrophages and SMCs in the majority of atherosclerotic lesions. As has been reported earlier [32], iNOS expression was also seen in some sections on endothelial cells (Fig. 2C). However, systematic analysis of endothelial iNOS expression was hampered by the absence or detachment of endothelial layer in the immersion-fixed tissue samples. Although in situ hybridization studies do not allow any quantitative assessments of mRNA levels, our studies gave the impression that the expression of iNOS mRNA tended to correlate with the number of macrophages. Also, the highest expression level was often detected in macrophages. Expression of iNOS in atherosclerotic lesions can be induced in macrophages and SMCs by tumor necrosis factor-α, interleukin-1 or γ-interferon [1, 2, 4, 33], all of which are present in atherosclerotic lesions [34]. Thus, iNOS expression in atherosclerotic lesions may

parallel activation of macrophages and SMCs by various proinflammatory factors.

It is not clear whether iNOS induction in atherosclerotic lesions is protective or whether it enhances lesion formation. NO produced by iNOS might have several antiatherogenic effects [18]; it can inhibit SMC proliferation [35] and thrombus formation, and since NO is an effective chain-breaking antioxidant, it can prevent LDL oxidation [36]. However, a high concentration of NO produced by iNOS can have cytotoxic effects on arterial cells, cause apoptosis and further induce the expression of proinflammatory genes [1, 18, 36]. According to recent studies, the net effect of increased NO production seems to be antiatherogenic, since dietary supplementation with L-arginine reduces lesion formation [37] and inhibition of NO synthesis enhances lesion formation [38, 39]. It is also important to note that in addition to increased iNOS expression during lesion development, superoxide anion production is stimulated by hypercholesterolaemia in endothelial cells [16], which can lead to increased peroxynitrite formation. Peroxynitrite can rapidly oxidize lipoproteins and cause several harmful effects in the arterial wall [9, 14, 40, 41]. Our results show that apart from two type-II lesions, epitopes characteristic of ox-LDL and peroxynitrite-modified proteins were *both* present in lesion areas positive for iNOS mRNA expression. Thus, it is likely that iNOS expression in atherosclerotic lesions is associated at least to some extent with localized oxidative damage to arterial cells and lipoproteins. Although it is not possible to prove that NO produced by iNOS and superoxide anion contribute to the formation of nitrotyrosine residues, our results confirm and extend previous reports [30, 42] of the presence of nitrotyrosine-modified proteins in atherosclerotic lesions. Thus, it is likely that peroxynitrite or other nitrating species are formed in atherosclerotic lesions.

Several types of oxidative injuries occur during atherogenesis, since malondialdehyde-, hydroxynonenal- [43] and peroxynitrite-modified proteins can be detected in human atherosclerotic lesions. The expression of iNOS mRNA is low in normal arteries, but we find that it increases substantially during lesion development. Both macrophages and SMCs express iNOS in the majority of different types of human atherosclerotic lesions. iNOS expression and oxidative injuries may play important parts in atherogenesis.

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