Expression of Extracellular SOD and iNOS in Macrophages and Smooth Muscle Cells in Human and Rabbit Atherosclerotic Lesions

Colocalization With Epitopes Characteristic of Oxidized LDL and Peroxynitrite-Modified Proteins

Jukka S. Luoma, Pontus Strålin, Stefan L. Marklund, Timo P. Hiltunen, Terttu Särkioja, Seppo Ylä-Herttuala

Abstract—Oxidative processes play an important role in atherogenesis. Because superoxide anion and nitric oxide (NO) are important mediators in vascular pathology, we studied the expression of extracellular superoxide dismutase (EC-SOD) and inducible nitric oxide synthase (iNOS) in human and rabbit atherosclerotic lesions by using simultaneous in situ hybridization and immunocytochemistry and EC-SOD enzyme activity measurements. We also analyzed the presence in the arterial wall of oxidized lipoproteins and peroxynitrite-modified proteins as indicators of oxidative damage and possible mediators in vascular pathology. EC-SOD and iNOS mRNA and protein were expressed in smooth muscle cells and macrophages in early and advanced lesions. The expression of both enzymes was especially prominent in macrophages. As measured by enzyme activity, EC-SOD was the major SOD isoenzyme in the arterial wall. EC-SOD activity was higher in highly cellular rabbit lesions but lower in advanced, connective tissue–rich human lesions. Despite the abundant expression of EC-SOD, malondialdehyde-lysine and hydroxynonenal-lysine epitopes characteristic of oxidized lipoproteins and nitrotyrosine residues characteristic of peroxynitrite-modified proteins were detected in iNOS-positive, macrophage-rich lesions, thus implying that malondialdehyde, hydroxynonenal, and peroxynitrite are important mediators of oxidative damage. We conclude that EC-SOD, iNOS, and the balance between NO and superoxide anion play important roles in atherogenesis. EC-SOD and iNOS are highly expressed in lesion macrophages. High EC-SOD expression in the arterial wall may be required not only to prevent deleterious effects of superoxide anion but also to preserve NO activity and prevent peroxynitrite formation. Modulation of arterial EC-SOD and iNOS activities could provide means to protect arteries against atherosclerotic vascular disease. (Arterioscler Thromb Vasc Biol. 1998;18:157-167.)

Key Words: SOD n inducible nitric oxide synthase n macrophages n oxidized LDL n peroxynitrite

Oxidized lipoproteins, reactive oxygen species, NO, and their reaction products are important mediators in vascular pathology.1–4 Oxidative stress regulates gene expression and may directly damage lipids and proteins.5 The arterial endothelium and some other cell types produce superoxide anion, the production of which is higher in hypercholesterolemia and under inflammatory conditions.6 SODs protect arteries and other tissues from the deleterious effects of superoxide anion and its reaction products.2,7 Three SODs have been cloned and characterized: cytosolic CuZnSOD,9 mitochondrial MnSOD,10 and EC-SOD, which contains a signal peptide for secretion and a heparan sulfate–binding domain.11 Cytosolic and mitochondrial forms of SOD constitute the majority of SOD activity in most tissues. In the arterial wall, however, EC-SOD activity is approximately 10-fold higher than in other tissues and is present in roughly the same amounts as CuZnSOD activity.8,12 Because the arterial wall is rich in connective tissue, it is likely that EC-SOD is bound to the extracellular matrix13 and constitutes an important defense mechanism against superoxide anions in the arterial microenvironment.

NO is an important regulator in vascular biology.14–16 NO produced by the endothelium modulates vascular tone and inhibits SMC proliferation and platelet aggregation.14–16 NO can suppress lipoprotein oxidation17–20 and inhibit lipid peroxidation by terminating radical chain reactions.21 On the other hand, NO availability in atherosclerotic arteries may be less-
oxidative processes in the pathogenesis of atherosclerosis. The findings suggest important roles for these enzymes and oxidized lipoproteins and peroxynitrite-modified proteins. In macrophage-rich areas colocalizes with the presence of atherosclerotic lesions and that the expression of these enzymes in macrophages express EC-SOD and iNOS in human and rabbit atherosclerotic arteries and to see whether their expression has anything to do with cellular damage, inflammation, and apoptosis.

Reaction of NO with superoxide anion occurs very rapidly and produces highly reactive peroxynitrite (ONOO\(^-\), which can directly damage lipids and proteins. \(^2\) It is evident that the balance between NO and superoxide anion in the arterial wall can have important consequences for arterial pathology and that this balance may change, depending on the inflammatory activation of the vascular cells. Although the presence of iNOS in mouse macrophages has been clearly demonstrated, \(^14-16\) it has been difficult to demonstrate the expression of iNOS in human macrophages. \(^23-25\) Also, it is not known which cell types express EC-SOD in normal and atherosclerotic arteries and whether there is any relationship between EC-SOD and iNOS expression and the presence of oxidized lipoproteins and peroxynitrite-modified proteins in atherosclerotic lesions.

The purpose of the present study was to analyze which cell types express EC-SOD and iNOS in human and rabbit atherosclerotic arteries and to see whether their expression has any relationship to the presence of epitopes characteristic of oxidative damage. We found that both SMCs and macrophages express EC-SOD and iNOS in human and rabbit atherosclerotic lesions and that the expression of these enzymes in macrophage-rich areas colocalizes with the presence of oxidized lipoproteins and peroxynitrite-modified proteins. The findings suggest important roles for these enzymes and oxidative processes in the pathogenesis of atherosclerosis.

### Methods

#### Tissue Samples

Human aortic samples were collected from medicolegal autopsies 3 to 14 hours postmortem (five men, aged 34, 41, 52, 73, and 79 years; two women, aged 35 and 51 years; a total of 25 aortic samples; Table 1). Tissue samples for paraffin-embedded sections were immediately transferred to formal/sucrose at 4°C (4% paraformaldehyde, 15% sucrose, 1 mmol/L EDTA, and 50 mmol/L BHT, pH 7.4). \(^26\) After fixation for 4 hours, the samples were rinsed overnight in 15% sucrose containing 1 mmol/L EDTA and 50 mmol/L BHT, embedded in paraffin, and used for in situ hybridization and immunocytochemical studies as described below. Tissue samples for frozen sections were immediately embedded in OCT compound (Miles Scientific), frozen, and kept at −70°C until analyzed. We cannot exclude the possibility of postmortem changes in the tissue samples. However, similar tissue samples in previous studies had shown no major changes when compared with results obtained from organ donor or perfusion-fixed animal tissue. \(^20-23\)

Sixteen aortic samples were collected from four WHHL rabbits (aged 7 to 50 months) and three 1% cholesterol–fed NZW rabbits (Table 1). Rabbits were anesthetized with intramuscular midazolam (2 mg/kg body weight) and fentanyl-fluanisone (0.5 mL/kg body weight), exsanguinated, and perfused for 5 minutes with physiological saline, followed by perfusion-fixation with formal/sucrose for 10 minutes. For frozen sections the aortic tissue was immediately removed, embedded in OCT, frozen, and kept at −70°C until analyzed. \(^26\) For paraffin-embedded sections, WHHL and NZW rabbit samples underwent additional immersion-fixation for 4 hours. Tissue samples were then kept overnight in 15% sucrose containing 1 mmol/L EDTA and 50 mmol/L BHT and embedded in paraffin. Serial sections (5 to 7 μm) were used for in situ hybridization and immunocytochemistry as described below. Human and rabbit aortic samples were classified according to Stary et al. \(^29\) as normal areas, type II (fatty streaks), type III (intermediate lesions), type IV (atheroma), type Vc (fibrous cap), type VI (atherosclerotic fibrous plaque).

### TABLE 1. Human and Rabbit Atherosclerotic Lesions Used for Immunocytochemistry and In Situ Hybridization Studies

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue Sample No.</th>
<th>Tissue Classification</th>
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<td>16</td>
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Sections were hybridized with riboprobes and oligonucleotide probes against EC-SOD and iNOS and were classified according to Stary et al. \(^29\) Plus indicates positive for EC-SOD or iNOS mRNA expression; minus, no detectable EC-SOD or iNOS mRNA expression; NA, not analyzed; NZW, NZW rabbits on 1% cholesterol chow for 8 weeks.

*Same block also contained adjacent normal intima, which was positive for EC-SOD and negative (or not analyzed) for iNOS.
In situ Hybridization
Antisense riboprobes and oligonucleotide probes for EC-SOD, CuZnSOD, and iNOS with the following antisense sequences were used for in situ hybridization: a human EC-SOD riboprobe (nucleotides 1018 to 1209), a rabbit EC-SOD oligonucleotide probe (nucleotides 175 to 219), a rabbit EC-SOD riboprobe (nucleotides 1 to 340), a human CuZnSOD oligonucleotide probe (nucleotides 373 to 417), a rabbit CuZnSOD riboprobe (nucleotides 243 to 469), and a mouse iNOS riboprobe (nucleotides 201 to 1071). Homology between mouse iNOS and human iNOS is 78%. Homology with rabbit iNOS is not known, but in a Northern blot, the mouse iNOS probe recognized a cytokine-inducible iNOS in rabbit alveolar macrophage mRNA and in rabbit arterial mRNA samples (data not shown). Corresponding sense riboprobes and sense oligonucleotide probes were used as controls for in situ hybridization analysis. Oligonucleotide probes were end labeled with 35 S-ATP (1000 to 1500 Ci/mmol, New England Nuclear) using terminal deoxynucleotidyl transferase. For riboprobe synthesis, the cDNAs were subcloned in pGEM (Promega Biotech) or pBluescript (Stratagene) vectors using standard techniques. Antisense and sense riboprobes were synthesized using T3-, T7- or Sp6-RNA polymerases with 35 S-UTP (1000 to 1500 Ci/mmol, New England Nuclear) as described. All reagents used for riboprobe synthesis were supplied by Promega Biotech. In situ hybridization studies were done on a set of serial sections as described. In brief, paraffin-embedded tissue sections were deparaffinized and rehydrated. Sections were treated with proteinase K, acetylated, dehydrated, and dried in vacuoum. Frozen sections were thawed, fixed with formal/sucrose for 10 minutes, dehydrated, and dried in vacuoum. Hybridization solution (50 μL) containing 6×10{sup}{sup}6 cpm/mL of each labeled probe was added to tissue sections and the sections were hybridized at 52°C for 14 hours. Hybridization solution contains 50% formamide (Fluka), 2× SSC, 20 mmol/L Tris, pH 7.4, 1× Denhardt’s solution, 1 mmol/L EDTA, 10% dextran sulfate (Pharmacia Biotech), 1 mmol/L DTT, and 0.5 mg/mL yeast tRNA (Boehringer-Mannheim Biochemicals). (1× SSC contains 150 mmol/L NaCl and 15 mmol/L sodium citrate, pH 7.0; Denhardt’s solution contains 0.02% Ficoll and 0.02% BSA [all from Sigma Chemical Co]). After hybridization the sections were washed three times (once for 30 minutes and twice for 5 minutes each) in 4× SSC at 37°C. The sections were then washed at 37°C in 2× SSC and 1× SSC (15 minutes each). The final wash for riboprobes was at 55°C in 0.1× SSC for 30 minutes. For the oligonucleotide probes the final wash was at 42°C in 1× SSC for 15 minutes. Tissue sections were then dehydrated, dried, dipped in autoradiographic emulsion (NTB-2, Eastman-Kodak Co), and developed after 2 to 10 weeks’ exposure time. After development the sections were counterstained with hematoxylin/eosin. Nonhybridizing sense riboprobes or oligonucleotide probes were used as controls. For some antibodies, immunocytochemistry was performed on the same sections before they were dipped into the autoradiographic emulsion.

Immunocytochemistry
Serial paraffin and frozen sections were used for immunocytochemistry. Immunostaining was done using the following antibodies: mouse mAb against human macrophages (HAM-56, DAKO), mouse mAb against SMCs (HHF-35, Enzo Diagnostics), mouse mAb against rabbit macrophages (RAM-11, DAKO), mouse mAb against iNOS (clone 6, Transduction Laboratories), guinea pig polyclonal antisera against MDA-LDL (MAL-2) and HNE-LDL (HNE-7), rabbit polyclonal antisera against bovine cytosolic CuZnSOD (The Binding Site), goat polyclonal antisera against human recombinant EC-SOD and mouse mAb (clone 1A6), and rabbit polyclonal antisera against nitrotyrosine residues. The avidin-biotin–horseradish peroxidase system (Vector Laboratories Inc) was used for signal detection. Complex for immunostaining included incubations wherein primary antibodies were replaced by irrelevant class- and species-matched antibodies and incubations wherein primary antibodies were omitted. The specificity of nitrotyrosine immunostaining was confirmed by blocking the staining with 10 mmol/L 3-nitrotyrosine.

SOD Activity Analysis
Frozen arterial sections were pulverized in a Braun Microdismembrator II (B Braun Biotech Inc), and the frozen powder was added to volumes of 50 mmol/L potassium phosphate, pH 7.4, with 0.3 mol/L KBr and a set of antiproteolytic agents (0.5 mmol/L PMSF, 3 mmol/L EDTA, 90 mg/L aprotinin, and 10 mg/L each of peptatin, chymostatin, and leupeptin; Sigma). The homogenates were then sonicated and finally centrifuged (20 000 g for 15 minutes). Unless analyzed immediately, the supernatants were stored at −80°C. The SOD enzymatic activity was determined by a direct spectrophotometric method employing KO2 as described. To distinguish between the cyanide-sensitive isoenzymes (CuZnSOD and EC-SOD) and the resistant one (MnSOD), 3 mmol/L cyanide was used. One unit in the assay is defined as that activity that causes a decay in superoxide anion concentration at a rate of 0.1/s in 3 mL of reaction buffer. In this assay 1 unit corresponds to 8.3 ng of human CuZnSOD, 6.3 ng of bovine CuZnSOD, 8.6 ng of human EC-SOD, and 65 ng of bovine MnSOD. The assay is carried out at pH 9.5 using a relatively high superoxide anion concentration. In comparison, the xanthine oxidase-cytochrome c assay, which is also used for SOD measurements, is carried out under more physiological conditions (i.e., neutral pH and low superoxide anion concentration). One unit in the current assay corresponds to ~0.024 U of CuZnSOD and EC-SOD and 0.24 U of MnSOD in the xanthine oxidase assay. Thus, the current assay is about 10 times more sensitive for CuZnSOD and EC-SOD activity than for MnSOD activity.

Specific Analysis of EC-SOD
EC-SOD in human artery wall extracts was determined by ELISA. There is no cross-reactivity with human CuZnSOD. For conversion of results to activity units, 8.6 ng per unit was assumed. For the specific analysis of EC-SOD in artery extracts from the rabbits, chromatography on concanavalin A–Sepharose (Pharmacia Biotech) was used. Unlike CuZnSOD and MnSOD, the glycoprotein EC-SOD binds to the lectin concanavalin A. The procedure has been described previously, the only difference being that the extraction buffer described above was used as a solvent in all steps. The yield of EC-SOD in the procedure was tested with human blood vessel extracts. Much (75%) of the applied EC-SOD was found to be recovered as determined by ELISA, and all EC-SOD results from the rabbits were corrected accordingly. The CuZnSOD activity of the extracts was then calculated as total cyanide-sensitive SOD activity minus (corrected) EC-SOD activity.

Protein and DNA Analyses
For protein analysis, Coomassie Brilliant Blue G-250 (Biorad) was employed, after it was standardized with human serum albumin. DNA concentration was determined by a fluorometric assay as a complex with bisbenzimidazole (Hoechst 33258) using calf thymus DNA as the standard.
Statistical Analyses

All data are expressed as mean±SD. Because we cannot guarantee a normal distribution of the results, comparisons of pooled WHHL rabbit samples from the aortic arch and thoracic aorta and from human arteries were made by using the Mann Whitney U test and Wilcoxon’s signed rank test, respectively. BMDP statistical software was used in all tests.

Results

EC-SOD mRNA expression in rabbit aortas was studied in 16 different sets of tissue samples, including fatty streaks, intermediate lesions, atheromas, and plaques. EC-SOD expression was detected in every normal and atherosclerotic aorta except in one early fatty streak, which was negative for EC-SOD mRNA (Table 1). Fig 1A through 1F shows representative examples of EC-SOD mRNA expression in serial sections of early and advanced lesions from WHHL rabbit thoracic aortas. Fig 1A shows immunostaining against macrophages using RAM-11 antibody. A strong expression of EC-SOD mRNA was seen in the macrophage-rich area (Fig 1B). Some expression was also seen in medial SMCs. Fig 1C and 1D are controls for immunocytochemistry and in situ hybridization, respectively. Fig 1E shows strong EC-SOD mRNA expression in subendothelial macrophages and in medial SMCs (Fig 1F) from a 2-year-old WHHL rabbit aortic arch; g and f, Serial sections of an early atherosclerotic lesion (type II) from a 1-year-old WHHL-rabbit thoracic aorta: e, in situ hybridization with a 35S-ATP end-labeled EC-SOD oligonucleotide probe showing the expression of EC-SOD in subendothelial macrophages and in medial SMCs (Fig 1G); and f, immunostaining for macrophages (RAM-11; dilution, 1:1000; original magnification, ×12.5); and f, control for in situ hybridization using a nonhybridizing sense probe (original magnification, ×12.5). g and f, Serial sections of an advanced atherosclerotic lesion (type IV) from a 2-year-old WHHL rabbit aortic arch: g, immunostaining for subendothelial macrophages (RAM-11; dilution, 1:1000; original magnification, ×12.5); h, in situ hybridization with a 35S-ATP end-labeled CuZnSOD oligonucleotide probe showing that CuZnSOD mRNA is expressed in subendothelial macrophages and in medial SMCs (original magnification, ×31.25); and i, control for in situ hybridization using a nonhybridizing sense probe (original magnification, ×31.25). Panels b, d, e, h, and i were photographed using polarized light epiluminescence. Hematoxylin and eosin counterstaining.
sion in an advanced WHHL rabbit lesion. Fig 1G shows immunostaining for macrophages. It is evident from Fig 1H that CuZnSOD mRNA is expressed in subendothelial macrophages and medial SMCs. Fig 1I is a control for in situ hybridization. CuZnSOD immunoreactive protein was also present in the lesions (data not shown).

EC-SOD mRNA and protein expression in human aortas was studied in 25 different sets of tissue samples, including fatty

Figure 2. EC-SOD mRNA and protein are expressed in macrophages and SMCs in human atherosclerotic lesions. Representative photomicrographs from three different human atherosclerotic lesions are shown. a-f, Serial sections of an advanced atherosclerotic lesion (type V) from the thoracic aorta of a 79-year-old man: a, a low-magnification view of the lesion (original magnification, ×12.5); b, in situ hybridization using a 35S-UTP-labeled human EC-SOD riboprobe, with simultaneous immunostaining for human macrophages (HAM-56; dilution, 1:500) showing the expression of EC-SOD mRNA in macrophages (arrowheads; original magnification, ×125); c, immunostaining for EC-SOD protein in the same area (goat antiserum; dilution, 1:500; original magnification, ×125); d, in situ hybridization using a 35S-UTP-labeled human EC-SOD riboprobe, with simultaneous immunostaining for SMCs (HHF-35; dilution, 1:250; dark-field image) showing the expression of EC-SOD mRNA as bright spots in the subendothelial and deeper parts of the lesion (original magnification, ×31.25); e, same section as in d under bright-field illumination showing mRNA as black spots and the distribution of SMCs under intermediate magnification (×62.5); and f, same section as in d, but at high magnification (×125) showing EC-SOD mRNA expression in SMCs (arrowheads). g-i, Serial sections of an early lesion (type II) from the aorta of a 51-year-old woman: g, control for in situ hybridization, using a nonhybridizing sense riboprobe (original magnification, ×31.25); h, in situ hybridization using a 35S-UTP-labeled human EC-SOD riboprobe, with simultaneous immunostaining for human macrophages (HAM-56; dilution, 1:500) showing the expression of EC-SOD mRNA in early lesion macrophages (arrowheads; original magnification, ×125); and i, in situ hybridization using a 35S-UTP-labeled human EC-SOD riboprobe, with simultaneous immunostaining for SMCs (HHF-35; dilution, 1:200) showing the expression of EC-SOD mRNA in early lesion SMCs (arrowheads; original magnification, ×125). j-l, Serial sections of an advanced lesion (type V) from the aorta of a 73-year-old man: j, immunostaining for EC-SOD protein (goat antiserum; dilution, 1:200; original magnification, ×62.5); k, same section as in j but at higher magnification, showing both cellular and extracellular staining pattern for EC-SOD protein (original magnification, ×125); and l, nonimmune control for the immunostaining (first antibody omitted; original magnification, ×31.25). Panels d and g were photographed using polarized light epiluminescence. Hematoxylin and eosin counterstaining.
TABLE 2. SOD Enzyme Activities and DNA and Protein Contents in WHHL Rabbit and Human Aortas

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<th>Atherosclerotic Lesions,* Mean±SD</th>
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WW indicates tissue wet weight. Tissue samples (intima-media) were dissected from the aortic arch and thoracic aortas of 1–2-year-old WHHL rabbits and from the aortas of 34–79-year-old humans. WHHL rabbit comparisons were made using the Mann-Whitney U test and human comparisons by a Wilcoxon signed rank test.

*Histology: Rabbit lesions were highly cellular and contained both macrophages and SMCs. Human lesions were less cellular, containing more connective tissue than the rabbit lesions.

staining with both cellular and extracellular staining patterns is shown in Fig 2J and 2K. A nonimmune control for the immunostaining is shown in Fig 2L.

To further study the expression of EC-SOD in atherosclerotic lesions, EC-SOD, CuZnSOD, and MnSOD enzyme activities were measured in WHHL rabbit and human lesions (Table 2). It was found that EC-SOD enzyme activity in WHHL rabbit lesions was significantly higher than in macroscopically normal aortas (P=.02). We did not find any differences in CuZnSOD or MnSOD activities between normal and atherosclerotic aortas (Table 2). DNA and total protein contents were similar in lesion and nonlesion areas. Histologically the analyzed lesions were highly cellular, containing both macrophages and SMCs (data not shown). Enzyme activity measurements are in line with in situ hybridization results, which showed a high level expression of EC-SOD mRNA in rabbit lesions.

In advanced human lesions, EC-SOD, CuZnSOD, and MnSOD enzyme activities differed from those in rabbit lesions (Table 2): EC-SOD and CuZnSOD enzyme activities were lower than those in macroscopically normal aortas. MnSOD activity did not show any change between lesion and nonlesion areas. However, the amount of DNA was lower in lesions than in normal aortas, indicating a decrease in the number of cells in advanced human lesions. The total protein content was also lower in lesions than in normal aortas. Histologically the analyzed human lesions were less cellular than the rabbit lesions. The results are in line with in situ hybridization results, which (though by no means quantitative) showed much less EC-SOD hybridization in human lesions than in rabbit lesions.

The expression of iNOS mRNA was also studied in the same rabbit (10 tissue samples) and human (14 tissue samples) aortas (Table 1). In rabbit lesions, macrophage-rich areas in all but two plaques showed strong expression of iNOS mRNA. iNOS expression was also detected in SMCs within and under the lesions. No expression of iNOS was found in normal rabbit arteries (Table 1). The expression of iNOS mRNA in macrophage-rich areas colocalized with epitopes characteristic of oxidized LDL. Fig 3 shows a representative example of a WHHL rabbit advanced lesion: In situ hybridization and immunocytochemistry show strong expression of iNOS mRNA and protein in the same area that contains macrophages and SMCs (data not shown). Immunocytochemistry of a serial section using an antibody against SMCs suggests that iNOS is also expressed in the SMCs (Fig 3D). Immunostaining with antisera against MDA-lysine (data not shown) and HNE-lysine epitopes characteristic of oxidized LDL (Fig 3F) show that oxidized LDL is localized to the same areas as iNOS mRNA and macrophages. The same areas also contain nitrotyrosine residues (Fig 3E). As shown above, similar lesions were highly positive for EC-SOD mRNA.

Similar results were obtained from human aortic samples (Table 1): macrophages and SMCs in all but one lesion expressed iNOS mRNA and protein and immunostained positively for epitopes characteristic of oxidized LDL. The negative lesion was an advanced atherosclerotic plaque with a low number of macrophages. Macrophage-rich areas were positive for nitrotyrosine immunostaining, indicating the production of peroxynitrite in the lesion area. A representative
example of a human atherosclerotic plaque is shown in Fig 4. In situ hybridization (Fig 4A) showed iNOS mRNA expression in the lesion area. Simultaneous immunostaining with antibodies against macrophages (Fig 4B–C) and SMCs (Fig 4D) indicated that iNOS mRNA is expressed in both cell types. These same areas were also positive for iNOS protein (Fig 4E), epitopes characteristic of oxidized LDL (Fig 4F), and nitrotyrosine residues (Fig 4G). The specificity of the nitrotyrosine immunostaining was confirmed by blocking the staining with 10 mmol/L 3-nitrotyrosine (data not shown). The strongest expression for iNOS mRNA was always seen in macrophage-rich areas.

**Discussion**

The current study shows that in the arterial wall, EC-SOD is expressed in SMCs and macrophages. Based on in vitro studies, it has been previously suggested that SMCs are the principal source of EC-SOD in the arteries. This appears to be true in normal arteries. However, our results show that a significant portion of EC-SOD mRNA in atherosclerotic lesions originates from macrophages. Because EC-SOD is a secreted enzyme with a high binding affinity for matrix components, immunocytochemistry alone cannot be used to localize cells that synthesize the enzyme, but additional in situ hybridization is needed to identify cells that express EC-SOD mRNA. The situation is similar to that of some other secreted proteins, such as lipoprotein lipase, which is expressed in parenchymal cells although the immunoreactive protein is mainly located on the endothelial surface. In vitro, it has been difficult to show EC-SOD expression in cells of the monocytic lineage. This may be due to the difficulty of finding optimal in vitro conditions for induction of EC-SOD activity in macrophages, a situation very similar to the difficulty of showing iNOS expression in human macrophages.

EC-SOD activity showed a significant increase in highly cellular rabbit atherosclerotic lesions, but cytosolic CuZn-
SOD or mitochondrial MnSOD did not show similar changes during lesion development. As reported previously, the overall EC-SOD activity in the arterial wall was approximately 10-fold higher than in extravascular tissues, where CuZnSOD and MnSOD constitute most of the total SOD activity. On the other hand, connective tissue–rich advanced human lesions showed decreased EC-SOD activity. A possible explanation for the differences in enzyme activities between human and rabbit lesions may be the differences in lesion biology and histology. The rabbit early lesions were highly cellular, with higher DNA and protein contents than in normal rabbit aortas, whereas advanced human lesions contained less DNA, fewer cells, and more connective tissue. The synthesis of EC-SOD by human fibroblasts is highly responsive to various inflammatory cytokines, although there is no response to oxidative stress. Human arterial SMCs respond similarly (P.S. and S.L.M., unpublished data, 1997). The differences in biology of the lesions may thus partially explain differences in EC-SOD contents. Finally, the regulation of EC-SOD synthesis may differ between humans and rabbits, and there may be differences in the amounts of heparan sulfate proteoglycans necessary for efficient retention of the secreted enzyme.

Our results demonstrate that iNOS is expressed in lesion macrophages and SMCs. iNOS expression can be induced by several factors, such as tumor necrosis factor-α, interleukin-1, and γ-interferon, all of which are present in atherosclerotic lesions. iNOS produces large quantities of NO, which can lead to cellular damage, inflammation, and apoptosis. Simultaneous in situ hybridization and immunocytochemistry studies confirmed the expression of iNOS mRNA in human macrophages, which has been difficult to demonstrate in vivo.

Although our results do not directly prove that iNOS-related NO and superoxide anion contribute to the forma-
tion of nitrotyrosine residues, the results strongly implicate superoxide anion in the inactivation of endogenous NO in macrophage-rich lesions. Our results confirm and extend previous reports of the presence of nitrotyrosine-modified proteins in atherosclerotic lesions and suggest that cytokine-induced macrophages express iNOS and produce NO, which can then react with superoxide anion, produce highly reactive peroxynitrite, and cause oxidative damage to cellular components and LDL. The rate for the reaction of superoxide anion with NO to produce peroxynitrite is very high \( [6.7 \times 10^{9} \text{ mol/L}^{-1} \text{s}^{-1}] \) and exceeds the rate of dismutation by SOD \( [2 \times 10^{7} \text{ mol/L}^{-1} \text{s}^{-1}] \). Because NO must move from the producer cells to the effector cells through the extracellular space, NO is susceptible to inactivation by several reactive compounds, such as superoxide anion and oxidized LDL. It can be calculated that when NO production by eNOS is maximally stimulated and when there is significant induction of iNOS, even the relatively high level of EC-SOD in the arterial interstitium is probably insufficient to prevent peroxynitrite formation. Thus, it is likely that peroxynitrite or other nitrating species are formed in atherosclerotic lesions. The concept is also supported by recent findings by White et al., who showed that administration of liposome-encapsulated CuZnSOD substantially improved arterial vasorelaxation by preventing the destruction of NO.

As shown previously, oxidized LDL is present in atherosclerotic lesions. Oxidized LDL can cause lipid accumulation in macrophages, monocyte recruitment into the intima, and various changes in the expression of proinflammatory genes. Hypercholesterolemia and the presence of lysolecithin in oxidized LDL have been shown to stimulate superoxide anion production by the endothelium. Because oxidized LDL can inhibit iNOS and inactivate NO, the antiatherogenic effects of NO and the ratio of NO to superoxide anion may be reduced in atherosclerotic arteries. All of these mechanisms may contribute to the increased consumption of NO and the oxidative damage in atherogenesis. It should be pointed out that in normal arteries, NO produced by eNOS probably plays an important antiatherogenic role by causing vasorelaxation and inhibiting SMC proliferation, platelet aggregation, and lipoprotein oxidation. Also, recent studies indicate that inhibition of NO synthesis promotes atherosclerosis, whereas supplementation with \( \alpha \)-arginine reduces atherogenesis. However, if conditions favor peroxynitrite formation, the situation could change dramatically, as may happen in macrophage-rich lesions. Other antiatherogenic effects of NO, such as inhibition of LDL oxidation and inhibition of the expression of adhesion molecules and monocyte chemotactic protein, could change accordingly.

We conclude that both SMCs and macrophages express EC-SOD and iNOS in human and rabbit atherosclerotic lesions. EC-SOD activity in atherosclerotic arteries is increased in highly cellular, rabbit lesions relative to the normal rabbit aorta but reduced in less cellular, advanced human lesions relative to the normal human aorta. High EC-SOD expression in the arteries and in activated macrophages may be required to protect the biological activity of NO. Macrophage-rich areas were positive for epitopes characteristic of oxidized LDL and peroxynitrite-modified proteins. This finding indicates that many types of oxidatively modified proteins are present in lesions and that multiple mechanisms are responsible for oxidative injuries in atherosclerotic arteries. The results also suggest that modulation of arterial wall EC-SOD and iNOS activities could be useful in the treatment of vascular disease.

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Expression of Extracellular SOD and iNOS in Macrophages and Smooth Muscle Cells in Human and Rabbit Atherosclerotic Lesions: Colocalization With Epitopes Characteristic of Oxidized LDL and Peroxynitrite-Modified Proteins

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