Cyclooxygenase-2 Is Widely Expressed in Atherosclerotic Lesions Affecting Native and Transplanted Human Coronary Arteries and Colocalizes With Inducible Nitric Oxide Synthase and Nitrotyrosine Particularly in Macrophages

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Abstract—Inflammation appears to have a major role in the development of atherosclerotic lesions affecting native and transplanted coronary arteries. The subsequent risk of plaque rupture and acute ischemic events correlates with the degree of inflammation and may be modified by aspirin, an anti-inflammatory cyclooxygenase inhibitor. Cyclooxygenase-2 (Cox-2) and inducible nitric oxide synthase (iNOS) are involved in the inflammatory response via the rapid and exaggerated production of prostanoids and nitric oxide, both of which may have proatherosclerotic effects. These effects may be mediated by the formation of peroxynitrite in the case of nitric oxide and involve “cross talk” between the two enzyme systems. This study aimed to investigate native and transplant atherosclerosis for the presence and distribution of Cox-2 and iNOS. Immunocytochemical studies were performed on atherosclerotic lesions from patients with native (n=12) and transplant (n=5) coronary disease by using antibodies to Cox-2, iNOS, and nitrotyrosine (an indicator of peroxynitrite production). Control tissue was obtained from unused donor hearts and at the time of autopsy. Cox-2 and iNOS colocalized predominantly in macrophages/foam cells in both types of atherosclerosis. Cox-2 expression was also detected in medial smooth muscle cells and endothelial cells, including those of the vasa vasorum. Nitrotyrosine was found in the same distribution as that of iNOS and was colocalized with Cox-2 in macrophages. Cox-2 and iNOS are coexpressed in native and transplant atherosclerosis, possibly allowing for interaction between the enzymes and suggesting an alternative mechanism for the benefits of aspirin via inhibition of Cox-2 activity. (Arterioscler Thromb Vasc Biol. 1999;19:646-655.)

Key Words: cyclooxygenase-2 ■ inducible nitric oxide synthase ■ atherosclerosis ■ transplant atherosclerosis ■ inflammation

Atherosclerosis is a major cause of death in Western civilizations. Current data support the hypothesis that atherosclerosis is an inflammatory disease,1,2 and studies examining markers of inflammation (eg, C-reactive protein) demonstrate a relation between increasing inflammation and risk of myocardial infarction.3,4 Plaque rupture leading to thrombosis is the key event in infarction and has been shown to be related to increased inflammation within the plaque rather than plaque morphology or degree of vessel stenosis.5 Furthermore, reduction in the inflammatory response may be associated with a reduction in the risk of subsequent ischemic events,2 and the beneficial effect of aspirin in reducing the risk of myocardial infarction has been suggested to be partly attributable to its anti-inflammatory action.3 In a similar manner, transplant atherosclerosis is the major long-term cause of death in cardiac transplant recipients6 and has been hypothesized to result from a chronic inflammatory reaction in response to repetitive immunological injury.7 Despite morphological differences, the 2 forms of atherosclerosis share many cellular similarities,8,9 including proliferation of smooth muscle cells and accumulation of macrophages and lymphocytes, and therefore share many theories relating to pathogenesis.

Cyclooxygenases (Coxs) and nitric oxide (NO) synthases (NOSs) exist as constitutive and inducible forms.10 The constitutive enzymes Cox-1 and endothelial NOS are generally regarded as being protective against atherosclerosis, because they inhibit platelet aggregation11,12 and smooth

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muscle proliferation and have beneficial effects on lipid metabolism and the immune response. The inducible enzymes Cox-2 and inducible NOS (iNOS) have catalytic actions similar to those of the constitutive forms. Cox-2 catalyzes the conversion of arachidonic acid to prostaglandin G2 (PGG2) and then to prostaglandin H2 (PGH2), the precursor for eicosanoid synthesis and iNOS converts l-arginine to l-citrulline with the release of NO. Both, however, are known to be involved in the pathogenesis of inflammatory disorders and are induced in response to a variety of inflammatory cytokines, many of which are known to be produced in both forms of atherosclerosis.

The damaging effect of the inducible enzymes appears to be caused by the amount and rapidity of release of prostanooids and NO. Large amounts of NO have been shown to lead to further inflammation, cellular damage, and apoptosis. This may be mediated by the formation of the highly reactive oxidant peroxynitrite from the reaction of NO with the superoxide anion. The production of peroxynitrite and other reactive nitrogen species may be inferred from the presence of nitrotyrosine, which is the stable, immunoreactive product of the reaction between peroxynitrite and tyrosine residues within proteins.

The possibility of significant “cross talk” between NO synthase and Cox has recently been suggested, and colocalization of Cox-2 and iNOS has been demonstrated in animal models of inflammation. NO has been shown to enhance Cox activity both in vitro and in vivo, and Cox activity can also produce the superoxide anion, thus providing the potential for peroxynitrite formation. It is noteworthy that peroxynitrite may also modulate eicosanoid synthesis, because it has been shown both to activate Cox-2 and to inactivate prostacyclin synthase. To our knowledge, neither Cox-2 expression nor its relation to iNOS has been demonstrated in either native or transplant atherosclerosis.

In view of the contrasting actions and interactions of Cox-2 and iNOS, the suspected role of inflammation in atherosclerosis, and the possible protective effect of aspirin, a Cox inhibitor, we hypothesized that Cox-2 and iNOS may be involved in the development and progression of atheroma. The aim of this study was 3-fold; ie, first, to determine the cellular location of Cox-2 in native and transplant atherosclerosis, second, to investigate for codistribution of Cox-2 and iNOS, and last, to examine for peroxynitrite formation in transplant atherosclerosis by using nitrotyrosine as a marker.

Methods

Tissues
Human epicardial coronary arteries were obtained, either from the left or from the right coronary circulation, from patients undergoing
cardiac transplantation for native vessel coronary artery disease (mean age, 49 years; range, 28 to 57 years; all male) \((n=12)\) or retransplantation for transplant atherosclerosis (mean age, 30 years; range, 13 to 46 years; 2 males, 3 females) \((n=5)\). Control tissue was obtained from unused transplant donor hearts \((n=2)\) and at the time of autopsy from patients dying of noncardiac causes \((n=3)\) (mean age, 42 years; 3 males, 2 females). Tissue was fixed within 2 hours of receipt for the donor hearts and within 12 hours of death when obtained at the time of the autopsy. This was a time scale similar to that for the diseased tissue, which had been collected between 1988 and 1996. Tissue was fixed in 10% (vol/vol) buffered formal saline and then embedded in paraffin blocks. Serial 3-\(\mu\)m sections were cut and floated in a water bath at 52°C before picking up onto coated slides (Vectabond, Vector Laboratories). Serial flip-flop sections were also cut for localization studies.

**Conventional Histology**

Sections were stained with hematoxylin and eosin for grading of the type and extent of atherosclerosis or for confirmation of histological normality of the control vessels.

**Antisera and Antibodies**

**Cox-2**

A rabbit polyclonal antiserum was raised against the unique 18–amino acid sequence from the carboxy-terminal portion of Cox-2 that does not react with Cox-1. This antibody has previously been characterized in rat smooth muscle cells.

**iNOS**

Two rabbit polyclonal antisera were used to immunostain for iNOS. The first (R2B4) was raised to a synthetic 25-residue peptide based

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**Figure 2.** Native vessel atherosclerosis demonstrated Cox-2 reactivity in macrophages/foam cells (A), smooth muscle cells (C), and endothelial cells (E). Cell type was determined by reactivity to CD68 (B), smooth muscle actin (D), and CD31 (F) in flip-flop sections. Brown reaction product indicates positivity. Magnification ×40 (A and B) and ×100 (C to F).
on the deduced amino acid sequence of cDNA encoding the human hepatocyte iNOS. The peptide corresponded to amino acid residues 47 to 71 of the human enzyme. The specificity of this antibody has previously been characterized in atherosclerosis. The second antiserum (SA-200) was the kind gift of Dr. J. Pollock and was raised to a 15-amino acid peptide again based on the human form of iNOS (Biomol Research Laboratories).

Nitrotyrosine
A polyclonal antiserum raised in rabbit against 3-nitrotyrosine was generated according to the method of Beckman et al in our laboratory (T.J. Evans).

To identify macrophages, endothelial cells, and smooth muscle cells, monoclonal antibodies to CD68, CD31, and smooth muscle actin, respectively, were obtained from DAKO Ltd (CD68 and CD31) and from Sigma.

Immunostaining
All sections were prepared for immunostaining by dewaxing in xylene and rehydrating to water through graded alcohols before autoclaving in 10 mmol/L sodium citrate buffer at pH 6 to retrieve antigen sites. The avidin–biotin–peroxidase complex method was used to stain sections. Endogenous peroxidase was blocked with 0.03% (vol/vol) hydrogen peroxide in methanol for 30 minutes followed by washing in 10 mmol/L PBS, pH 7.1 to 7.4 (3 washes of 5 minutes each). Nonspecific binding was blocked by incubation with 3% (vol/vol) normal goat serum for polyclonal antibodies and 3% (vol/vol) normal horse serum for monoclonal antibodies. Sections were blotted to remove excess serum and incubated overnight at 4°C with optimally diluted antisera to iNOS (R2B4, 1:2000; SA-200, 1:200), CD68 (1:25), CD31 (1:20), smooth muscle α-actin (1:16 000), and Cox-2 (1:2000). Sections were washed in PBS and then incubated for 45 minutes at room temperature with biotinylated

![Image of immunostain results](http://atvb.ahajournals.org/)}
goat antiserum to rabbit IgG or with horse antiserum to mouse IgG (Vector Laboratories), diluted 1:100. After further washes in PBS, freshly prepared avidin–biotin–peroxidase complex (Vectastain, Vector Laboratories) was applied for 45 minutes. Peroxidase activity was revealed with the glucose oxidase diaminobenzidine by using the nickel enhancement method\(^4\) for iNOS and with diaminobenzidine and hydrogen peroxide for the Cox-2, nitrotyrosine, α‐actin, and CD68. Hematoxylin or neutral red were used as counterstains. Sections were dehydrated, cleared, and mounted in DPX (Merck Ltd).

Sections immunostained for nitrotyrosine did not require autoclaving and were prepared by using the avidin–biotin–alkaline phosphatase complex method. Nonspecific binding was blocked with 3% (vol/vol) normal goat serum and then blotted. Sections were incubated overnight at 4°C with antiserum (1:25) and washed with PBS before the addition of biotinylated goat antiserum (1:200) to rabbit IgG for 45 minutes at room temperature. After rinsing in PBS, the sections were incubated for 45 minutes with Vectastain ABC alkaline phosphatase reagent (Vector Laboratories). Sections were then developed in fast red naphthol (Sigma) for 15 minutes, washed in PBS and then water, and counterstained with hematoxylin and mounted in Hydromount (Merck). This method was chosen for all but the flip-flop sections, rather than the avidin–biotin–peroxidase complex technique, as pilot studies suggested that blocking of nonspecific antibody binding with methanol and hydrogen peroxide adversely affected the immunostaining.

Control sections were immunostained as above but with omission of the primary antibody layer. Further specificity controls were made by immunostaining sections after overnight incubation of the primary antibody with the homologous antigen. For iNOS and Cox-2, this meant 10 mmol/L and 200 μg/mL, respectively, of the peptide used to raise the antibody, and for nitrotyrosine, 10 mmol/L of 3-nitro-L-tyrosine (Alexis Corp).

Cell-type location of Cox-2, iNOS, and nitrotyrosine and colocalization of Cox-2/iNOS, Cox-2/nitrotyrosine, and nitrotyrosine/iNOS were assessed from histological appearance and dual staining by using flip-flop serial sections.

**Results**

**Histology**

Sections of coronary arteries taken from control hearts showed no significant histological evidence of atherosclerosis. Advanced atheromatous lesions were seen in all 12 patients with native vessel disease (Figure 1A). These included calcification in 5 cases. Typical concentric lesions were seen in all subjects with transplant atherosclerosis (Figure 1B).

Immunoreactivity for Cox-2 was not detected in coronary arteries taken from unused donor hearts or in histologically normal arteries taken at the time of autopsy. Immunostaining in all cases of native vessel atherosclerosis was seen in macrophages/foam cells, intimal and medial smooth muscle cells, and endothelial cells as identified by antibodies to CD68, smooth muscle actin, and CD31 in flip-flop sections (Figure 2). Smooth muscle cells and endothelial cells of the vasa vasorum similarly demonstrated reactivity for Cox-2.

Immunoreactivity for Cox-2 was also seen in macrophages/foam cells, intimal and medial smooth muscle cells, and endothelial cells (Figure 3) in all cases of transplant atherosclerosis. Again, immunoreactivity to Cox-2 was seen in endothelial cells and medial smooth muscle cells of the vasa vasorum (Figure 4). Specificity of the antibody was

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**Figure 4.** Cox-2 immunoreactivity also localized to the endothelial (A) and smooth muscle cells (C) of the vasa vasorum as identified by staining with CD31 (B) and smooth muscle actin (D). Magnification x100.
supported by successful absorption with the relevant peptide (data not shown).
iNOS was not detected in coronary arteries taken from unused donor hearts or in histologically normal arteries taken at the time of autopsy. Immunoreactivity was seen in all cases of native atheroma, predominantly in macrophages/foam cells (Figure 5A and 5B), although a proportion of cases exhibited immunostaining of intimal and medial smooth muscle cells (data not shown). Homogenous material within the atherosclerotic plaque also immunostained for iNOS (Figure 6B).
Transplant atherosclerosis also immunostained for iNOS in macrophages/foam cells in all cases (Figure 5C) and intimal and medial smooth muscle cells in half the cases. Specificity of the iNOS antibody (SA-200) was confirmed by successful absorption with peptide (Figure 6).
Nitrotyrosine immunoreactivity was not detected in histologically normal arteries. Nitrotyrosine, however, was demonstrated in cells shown to express iNOS in both native and transplant atherosclerosis (Figure 7A and 7B). Specificity of the antibody was demonstrated by successful absorption of antiserum with 3-nitro-[scap]-tyrosine (Alexis Corporation) (data not shown).
Colocalization of Cox-2 with iNOS and nitrotyrosine was demonstrated predominantly in macrophages/foam cells by using dual staining of flip-flop sections from both native and transplant vessel atherosclerosis (Figure 7C and 7D).

Figure 5. Reactivity to iNOS (black) was localized predominantly to macrophages within the atherosclerotic plaque in both native (A) and transplant (C) related disease. Macrophages are identified by reactivity to CD68 (B) and iNOS is seen to colocalize with nitrotyrosine (D, red reaction product). Magnification ×20 (A and B) and ×40 (C and D).

Discussion
We have demonstrated the presence of Cox-2, iNOS, and nitrotyrosine immunoreactivity in native and transplant atherosclerosis. Cox-2 was found in several distinct cell types, most prominently in macrophages and foam cells but also within intimal and medial smooth muscle cells and in endothelial cells (including those of the vasa vasorum). iNOS immunoreactivity was also seen in macrophages and smooth muscle cells in transplant and native disease and was seen to colocalize with Cox-2. The codistribution of iNOS and Cox-2 with nitrotyrosine, predominantly within macrophages and foam cells, suggests the active production of NO and formation of peroxynitrite.
The finding of Cox-2 immunoreactivity is consistent with previous evidence showing excess prostanoid production in rabbit models of atherosclerosis and in patients with severe atheroma. Furthermore, macrophages expressing Cox-2 are known to produce eicosanoids that have proinflammatory effects, increasing vascular permeability, promoting chemotaxis, and favoring cell proliferation and cholesterol ester retention. Eicosanoids from activated endothelium can also be shown to promote monocyte adhesion and diapedesis. These are all actions that may contribute to both the formation and subsequent progression of atherosclerosis.
Hyperlipidemia is a risk factor for both native vessel and transplant atherosclerosis and appears to be mediated by oxidized LDLs (oxLDLs), which can lead to endothelial injury,
monocyte migration, foam cell formation, and altered synthesis of cytokines and growth factors. Recent evidence suggests that oxLDLs may mediate some of its actions via an increase in Cox-2 synthesis and subsequent excess prostaglandin production.\textsuperscript{46,47} In addition, monocyte adhesion stimulated by oxLDLs and by interleukin-1 appears to be Cox dependent.\textsuperscript{46,48}

Two recent pieces of evidence are of particular relevance to the role of Cox-2 in inflammation and atherosclerosis. The first is the suggestion that Cox-2 in activated human monocytes may be able to generate the prostaglandin 8-epi-PGF\textsubscript{2a},\textsuperscript{49} which was initially shown to be produced by the action of free radicals on arachidonic acid. This compound is both mitogenic, leading to cellular proliferation, and vasoactive, leading to vasoconstriction,\textsuperscript{50,51} and thus may play a role in the genesis of atherosclerosis. The second is that Cox-2, with its nuclear location, may produce eicosanoids that are

Figure 6. Hematoxylin and eosin–stained section of a native vessel atherosclerotic plaque (A) showing iNOS immunoreactivity (black reaction product) in macrophages (B, large arrow) and within homogenous material (B, small arrows). Specificity of the antibody was ascertained by overnight incubation of the antibody with peptide (C). Magnification $\times20$.\textsuperscript{652}
active within the nucleus and these eicosanoids may modulate transcriptional events.\textsuperscript{52}

We therefore hypothesize that the presence of Cox-2 demonstrated in both native and transplant atherosclerosis is likely to contribute to the development and progression of coronary artery disease. Previous attempts to affect the pathogenesis of atherosclerosis with nonsteroidal anti-inflammatory drugs and steroids, known to affect Cox-2 activity, have met with conflicting results.\textsuperscript{53} This is probably because of the varying specificity of nonsteroidal anti-inflammatory drugs for Cox-1 and Cox-2 and the conflicting actions of steroids on lipid metabolism. The finding of a primary preventive action of aspirin in reducing the risk of first myocardial infarction and the relation of this risk to inflammation, as assessed by C-reactive protein levels,\textsuperscript{3} along with our current findings requires further investigation of the precise role of Cox-2 in atherosclerosis.

A similar role for iNOS in atherosclerosis is based on several observed proatherosclerotic effects that appear to be caused by the excessive production of NO and the consequent formation of peroxynitrite from the reaction with the superoxide anion.\textsuperscript{26} Peroxynitrite is a powerful oxidant and can lead to the oxidation of LDLs,\textsuperscript{54} attenuation of normal NO-dependent vasorelaxation,\textsuperscript{54} direct oxidative damage to cellular components,\textsuperscript{55} and possible proproliferative effects.\textsuperscript{27} Peroxynitrite may also act via the direct production of prostaglandin derivatives from plasma lipids and LDLs in a way similar to Cox-2.\textsuperscript{56} Furthermore, NO and peroxynitrite are known to induce apoptosis,\textsuperscript{57} and recent evidence suggests that apoptosis is abundant in atherosclerosis and may contribute to the accumulation of atheromatous debris and plaque instability.\textsuperscript{58} The finding of iNOS immunoreactivity in homogenous material within the atheromatous plaque is likely to be a result of protein leakage from dead macrophages.

Despite the evidence supporting a detrimental role for iNOS and the colocalization with nitrotyrosine, our study does not specifically exclude the possibility of iNOS producing NO in a manner similar to endothelial NOS, with its associated antiatherosclerotic actions. Indeed, inhibition or deficiency of iNOS, particularly early after transplantation, may lead to an increase in allograft intimal thickening.\textsuperscript{59,60} It therefore appears all the more likely that the effect of iNOS expression is dependent on the milieu in which it occurs and may be altered by other enzymes and their products.

The demonstration of codistribution of iNOS and nitrotyrosine in native coronary atherosclerosis confirms the findings of recent studies published by our laboratory\textsuperscript{37} and others.\textsuperscript{27} Evans et al.\textsuperscript{61} however, found no evidence of tyrosine nitration in atherosclerosis affecting human aorta by using Western blotting and HPLC. The reasons for this discrepancy are not immediately clear, because Western blotting has previously shown nitrotyrosine in aortic atherosclerosis,\textsuperscript{27,37} although differences in the antibody type and concentration used exist between the studies. Our results also agree with two previous studies\textsuperscript{62,63} in finding iNOS in transplant atheroma.

\begin{figure}
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\caption{Macrophages within both native (A) and transplant (B) atherosclerosis showed reactivity to nitrotyrosine that colocalized with the expression of Cox-2 (compare distribution with serial sections shown in Figures 2A and 3A). iNOS expression also colocalized with Cox-2 (C and D). Magnification \times40.}
\end{figure}
The codistribution of iNOS and Cox-2, as was seen in both native and transplant atheroma, has been shown previously in a variety of cell types in vitro, although there is conflicting evidence as to whether NO stimulates or inhibits Cox-2 or whether prostanoids can influence NO output. The finding, however, does allow us to speculate on a direct interaction between eicosanoids and iNOS and/or NO and Cox-2 leading to an exacerbation of the inflammatory process in atherosclerosis. The finding of colocalization of Cox-2 and nitrotyrosine would allow for some of this interaction to be mediated via peroxynitrite, which, although it is an activator of prostaglandin synthase, prevents the beneficial subsequent formation of prostacyclin by nitrating a tyrosine near the active site of prostacyclin synthase.

In conclusion, this study has demonstrated the presence of Cox-2 in native and transplant atherosis. This suggests a mechanism by which aspirin reduces the risk of myocardial infarction and a possible future means of therapeutic intervention using new Cox-2–specific inhibitors. The codistribution of Cox-2, iNOS, and nitrotyrosine immunoreactivity in both types of atherosclerosis supports the hypothesis that Cox-2 and iNOS may interact in these disorders. This suggests the possible manipulation of NO or peroxynitrite production, using agents such as superoxide dismutase, to modulate the pathogenesis of native and transplant coronary artery disease.

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