Expression Changes of the Vascular Antioxidant System in Atherosclerotic Coronary Arteries

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Oxidative stress induced by reactive oxygen species (ROS) plays an important role in atherogenesis, and the redox state is determined by the balance between antioxidants and the ROS generating system. To defend against enhanced ROS, mammalian cells have a complex network of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase. To clarify the role of the vascular antioxidant system, we investigated by immunohistochemistry the expression changes of antioxidative enzymes in coronary arteries obtained from autopsied cases. In nonatherosclerotic coronary arteries, Cu/Zn SOD and Mn SOD were expressed in medial smooth muscle cells (SMC), whereas cytosolic GPx (GPx-1) was expressed mainly in endothelium and weakly in medial SMC. Catalase was expressed in medial SMC and endothelium. Progression of atherosclerosis did not result in an additional increase in the expression of antioxidative enzymes in SMC in the media or endothelium. However, migrating SMC and macrophages in atheromatous plaques expressed these four antioxidative enzymes intensively. Double staining with cell markers confirmed the cell-specific expression of the antioxidative enzymes. Thus, the expression pattern showed regional heterogeneity. In response to oxidative stress, the vascular antioxidant system was upregulated in atherosclerotic lesions. The imbalance between vascular antioxidant and oxidant systems might play an important role in coronary atherogenesis. J Atheroscler Thromb, 2002; 9: 184–190.

Key words: Superoxide, Atherosclerosis, Glutathione peroxidase, Catalase

Introduction

Coronary atherosclerosis is a chronic inflammatory disease of the arterial wall, and atherosclerotic plaques are associated with the production of reactive oxygen species (ROS), which are thought to induce oxidative stress. Oxidative stress plays a crucial role in atherogenesis through various mechanisms such as endothelial injury, oxidative modification of low-density lipoprotein (LDL).

induction of redox-sensitive genes, including monocyte chemoattractant protein (MCP)-1 and adhesion molecules such as vascular cell adhesion molecules (VCAM). The redox state in the vasculature is determined by the balance between pro-oxidants and antioxidants. Recently, it has become apparent that NADH/NADPH oxidase is one of the key enzymes as a source of ROS in the vessel wall (1). On the other hand, vascular cells have a complex antioxidant system to protect themselves against increased oxidative stress. The antioxidative system is controlled by nonenzymatic and enzymatic mechanisms. Glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase are important antioxidant enzymes that scavenge ROS against oxidative stress. Previous

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studies have demonstrated that these antioxidants are finely regulated by various cytokines and hemodynamic forces, however, their precise localization and distribution in human coronary arteries remain poorly understood. Examination of the expression of antioxidant enzymes in diseased vessels has demonstrated that Cu/Zn SOD, Mn SOD and catalase are confined primarily to the endothelium in the aortic wall of control rabbits, however, the expression of the enzymes in hypertensive and hyperlipidemic rabbits increased through the aortic walls, including the endothelium, and the media (2). Cytosolic GPx (GPx-1) protein is strongly expressed in the cytoplasm of the foamy cells in and around atheromatous plaques in human arteries, whereas the staining is almost in negative control cases (3). Thus, in atherosclerotic vessels, the expression of antioxidative enzymes may be dynamically regulated. To date, the localization and expression change of antioxidant enzymes in human coronary arteries has not been investigated. Therefore, the present study was undertaken to investigate the localization of four major antioxidant enzymes, GPx-1, Cu/Zn SOD, Mn SOD and catalase, in coronary artery specimens obtained at autopsy; and their expression changes in atherosclerotic coronary arteries were also examined.

Materials and methods

Human Tissue

Human coronary arteries, 6 nonatherosclerotic and 7 atherosclerotic segments, were collected at autopsy, from 13 cases (58 to 84 years old) within 5 hours of death. There were multiple causes of death, including various types of malignancy, and two cases involved cardiac death. The segments were cut into 3-mm long pieces, then serial sections were embedded in O.C.T. compound, snap-frozen in liquid nitrogen and stored at -80°C until use. The sections were cut into five-micron-thick rings, placed on microslides, air-dried, fixed with acetone for 10 minutes, air dried and subjected to immunohistochemistry and immunofluorescence.

Immunohistochemistry

Immunohistochemistry was carried out as described (4). Briefly, the sections were blocked with bovine serum albumin (DAKO LSAB kit, DAKO A/S, Glostrup, Denmark) for 30 minutes at room temperature and then incubated with a primary antibody overnight at 4°C. The primary antibodies were sheep polyclonal anti-GPx-1 (Biogenesis), sheep polyclonal anti-Cu/Zn SOD (Upstate Biochem.), mouse monoclonal anti-MnSOD (Chemicon) and rabbit anti-catalase (Calbiochem). The sections were washed with Tris-buffered solution and incubated with streptavidin-horseradish peroxidase conjugate (DAKO LSAB kit, DAKO A/S). For negative control, the primary antibody was replaced with a carrier nonspecific immunoglobulin.

Double-Labeling Immunofluorescence

To identify the cell types, the sections were blocked with bovine serum albumin (DAKO LSAB kit, DAKO A/S) for 30 minutes at room temperature and incubated together with the anti-GPx1 or the anti-Cu/Zn SOD antibody and antibodies for cell-specific markers overnight at 4°C. The cell-specific marker antibodies were mouse monoclonal anti-human CD68 (clone KP-1, DAKO) for macrophages, mouse monoclonal anti-human smooth muscle α-actin (clone 1A4, DAKO) for smooth muscle cells and mouse monoclonal anti-human von Willebrand factor (clone F8/86, DAKO) for endothelial cells. The sections were washed three times with 50mM Tris-based buffer, incubated with biotinated anti-sheep IgG (Chemicon) and FITC-conjugated anti-mouse immunoglobulin (Amersham Pharmacia Bio-From 1494tech) for one hour at room temperature, washed three times with Tris-based buffer and incubated for 20 minutes with Streptavidin Texas Red (Amersham Pharmacia). The sections were then examined by a laser scanning confocal imaging system (MRC-1024, Bio-Rad Laboratories).

Results

All the samples were divided into nonatherosclerotic and atherosclerotic coronary arteries according to the classification of the American Heart Association (5-7). Nonatherosclerotic coronary arteries showed only mild adaptive intimal thickness, whereas atherosclerotic coronary arteries demonstrated various pathological changes such as marked thickening of the intima with collagen deposition, infiltration of inflammatory cells, or deposits of lipid core.

Expression of Antioxidants in Nonatherosclerotic Coronary Arteries

Immunohistochemical examination of GPx-1, Cu/Zn SOD, Mn SOD and catalase in nonatherosclerotic coronary arteries showed that GPx-1 was localized predominantly in the endothelium and only weakly expressed in the medial smooth muscle cells (Fig. 1A). Cu/Zn SOD was expressed mainly in medial smooth muscle cells. Cu/Zn SOD had only weak immunoreactivity in the endothelium (Fig. 1B). The localization of Mn SOD was similar to that of Cu/Zn SOD (Fig. 1C). In contrast, catalase was expressed in both the endothelium and the media (Fig. 1D).

To identify the GPx-1- or Cu/Zn-SOD-expressing cells, double-staining immunofluorescence with cell-specific markers was carried out. Consecutive sections were stained with the anti-GPx1 antibody and the anti-von Willebrand factor antibody. As shown in Figs. 2A, B and C, GPx-1-expressing cells were co-stained with the anti-von Willebrand factor antibody. The immunoreactivity of Cu/Zn SOD was co-localized with smooth muscle α-actin (Figs. 2D, E, and F).
Expression of Antioxidants in Atherosclerotic Coronary Arteries

The progression of atherosclerosis did not result in any additional increase in the expression of GPx-1 in the endothelium or the media (Fig. 3), however, the inflammatory cells that accumulated in atheromatous plaques were strongly positive for GPx-1. Similar expression changes were observed for Cu/Zn SOD, Mn SOD and catalase (Fig. 3). Double-staining immunofluorescence with antibodies against smooth muscle α-actin or CD68 confirmed that migrating smooth muscle cells and infiltrating macrophages expressed catalase and Cu/Zn SOD in atherosclerotic coronary arteries (Fig. 4).

Discussion

Oxidative stress has been implicated in several steps leading to the development of atherosclerosis. To adapt to the increased oxidative stress, every type of cell in the artery wall has an antioxidative enzyme defense system composed of a variety of enzymes such as SOD, GPx, catalase, thioredoxin, and glutaredoxin. There are also several isoforms of each enzyme, which differ in their tissue distribution, their subcellular localization and the cofactors they require for catalytic activity. In nonatherosclerotic coronary arteries, GPx-1 was mainly expressed in the endothelial cells, whereas Cu/Zn SOD and Mn SOD were expressed predominantly in the medial smooth muscle cells. Catalase was intensively expressed in both endothelial and medial smooth muscle cells. Thus, these antioxidative enzymes were widely expressed in human coronary arteries in a predominantly heterogeneous manner. In atherosclerotic coronary arteries, the intensity of expression of the four antioxidative enzymes in the intima and the media was almost unchanged; however, the migrating smooth muscle cells and the infiltrating macrophages in atheromatous plaques expressed the enzymes intensively. Our observations indicated that each antioxidative enzyme defense system in the arteries has cell-specific heterogeneity. Our observations are quite consistent with previous reports on the expression of antioxidative enzymes in other vascular beds: Intensive GPx-1 expression is shown by foamy cells of atheromatous plaques in the aorta, but not in endothelial cells (3). Immunohistochemically, considerable amounts of antioxidative enzymes have been detected in early hypertensive and atherosclerotic arteries of the rabbit model compared with control animals (2). The activity of Cu/Zn SOD, Mn SOD, and GPx-1, as well as each of their protein concentrations, is lower in tissue
Fig. 2. Double staining with the von Willebrand factor, a marker of the endothelium (B), and α-actin (E), a marker of smooth muscle cells. The immunoreactivity of GPx-1 (A) and Cu/Zn SOD (D) was co-localized with that of the anti-von Willebrand factor (C) and α-actin (F), respectively.

Fig. 3. Immunohistochemistry showing expression changes of GPx-1, Cu/Zn SOD, Mn SOD, and catalase in atherosclerotic coronary arteries. GPx-1 (A, B and C), Cu/Zn SOD (D, E and F), Mn SOD (G, H and I), and catalase (J, K and L) were expressed intensively in infiltrating inflammatory cells in plaques.
of patients with aortic occlusive disease than in non-diseased control aortic tissue (8). Taken together with our findings, these results show that the expression of antioxidative enzymes is dynamically regulated against increased oxidative stress in atherosclerotic vessels.

SOD converts $O_2^*$ to $H_2O_2$, and the $H_2O_2$ so formed has atherogenic properties such as growth-promoting effects on smooth muscle cells (9) and enhancement of endothelial achesiveness (10). Since $H_2O_2$ is freely diffusible within and between cells, it is a key molecule that functions as an intracellular second messenger in response to extracellular stimuli. Early in vitro studies have demonstrated that tyrosine kinase and tyrosine phosphatases are targets of $H_2O_2$ (11, 12). Furthermore, there is evidence that $H_2O_2$ is involved in the activation of ERK1/2 and p38 MAPK (13, 14). SOD might influence these intracellular signal transductions by the modulation of $H_2O_2$. Of the three isoforms of SOD, Cu/Zn SOD, Mn SOD and extracellular SOD (EC-SOD), Cu/Zn SOD is located in the cytosol and Mn SOD in the mitochondria. In the present investigation, the expression pattern of these two SOD isoforms was very similar. In addition to these two isoforms, the human arterial wall contains EC-SOD that might have important implications in atherogenesis. EC-SOD is also expressed in macrophages in the atheroma of hypercholesterolemic animal models (15), however its functional role in human coronary atherosclerosis remains to be elucidated.

The cytotoxic effect of $H_2O_2$ is mediated by the production of a highly reactive hydroxyl radical (OH$^*$), which is generated by the Fenton reaction. A defense system against OH$^*$ does not exist in humans, therefore, the defense against $H_2O_2$ may be crucial in reducing oxidative stress. GPx as well as catalase can reduce $H_2O_2$ to $H_2O$. Furthermore, as a soluble selenoprotein, GPx reduces not only $H_2O_2$ but also organic hydroperoxides to $H_2O$ and corresponding alcohols. To date, five GPx isoenzymes have been identified: cytosolic GPx (GPx-1) and phospholipid hydroperoxide GPx (GPx-4 or PHGPx) are found in most tissues; gastrointestinal GPx (GPx-2) and extra-
cellular GPx (GPx-3) are localized in the gastrointestinal tract and kidney, respectively; and GPx-5, a selenium-independent GPx is specifically expressed in the mouse epididymis. In the present study, GPx-1 was localized mainly in the vascular endothelium of the coronary arteries. Endothelial cells are continuously exposed to blood flow and are the primary target of oxidant-induced injury. Laminar shear stress to cultured endothelial cells upregulates the expression of GPx-1 (16). Given the importance of blood flow in the regulation of endothelial function, GPx-1 is probably a primary defense system against H$_2$O$_2$ in the endothelium.

Catalase, another defense system against H$_2$O$_2$, was intensively expressed in the endothelium and medial smooth muscle cells in the coronary arteries. The overexpression of catalase inhibits proliferation of vascular smooth muscle cells and promotes their apoptosis, indicating that H$_2$O$_2$ mediates cellular proliferation, whereas O$_2^-$ induces apoptosis in vascular smooth muscle cells (17). Furthermore, H$_2$O$_2$ regulates the modulation of cellular phenotypes of smooth muscle cells (18). Thus catalase in coronary arteries might be a determinant of cellular proliferation, cell survival and phenotypic change of vascular smooth muscle cells.

Increased oxidative stress in the vascular wall is reported to contribute to the migration of vascular smooth muscle cells. On the other hand, macrophages have the ability to produce a large amount and are a major source of ROS in atherosclerotic vessels. Our study demonstrated that migrating SMC and infiltrating macrophages expressed the antioxidative enzymes tested intensively in atherosclerotic plaques. The mechanism whereby these two types of cells expressed the antioxidative enzymes remains to be elucidated; however, they may be counter-regulatory against enhanced oxidative stress. Inexpedient upregulation of antioxidative enzymes leads to local enhancement of oxidative stress, which, in turn, promotes further atherogenic processes.

In conclusion, the expresional pattern of antioxidative enzymes showed regional heterogeneity, and the expresion of each enzyme was cell-specific. In response to increased oxidative stress in atherosclerotic lesions, vascular antioxidant systems were upregulated in inflammatory cells. These observations suggest that the imbalance between vascular antioxidant and oxidant systems might play an important role in coronary atherogenesis.

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