Expression of NADH/NADPH Oxidase p22\textsuperscript{phox} in Human Coronary Arteries

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Background—NADH/NADPH oxidase is an important source of superoxide in the vasculature. Recently, we found that polymorphism of the gene p22\textsuperscript{phox}, a critical component of this oxidase, is associated with a risk of coronary artery disease. The aim of this study was to investigate the localization of p22\textsuperscript{phox} in human coronary arteries and to examine its difference in expression between nonatherosclerotic and atherosclerotic coronary arteries.

Methods and Results—Using coronary artery sections from autopsied cases (n = 11), the expression of p22\textsuperscript{phox} was examined by immunohistochemistry and Western blotting. In nonatherosclerotic coronary arteries, p22\textsuperscript{phox} was weakly expressed, mainly in the adventitia. In atherosclerotic coronary arteries, intensive immunoreactivity was detected in neointimal and medial smooth muscle cells and infiltrating macrophages in hypercellular regions and at the shoulder region. Semiquantitative analysis and Western blotting showed that the expression of p22\textsuperscript{phox} in atherosclerotic coronary arteries was more pronounced than that in nonatherosclerotic arteries. Double staining revealed p22\textsuperscript{phox} expression in adventitial fibroblasts, smooth muscle cells, macrophages in the neointima and media, and endothelial cells.

Conclusions—As atherosclerosis progressed, the expression of p22\textsuperscript{phox} increased through the vessel wall. p22\textsuperscript{phox} might participate in the pathogenesis and pathophysiology of atherosclerotic coronary disease. (Circulation. 1999;100:1494-1498.)

Key Words: atherosclerosis ■ free radicals ■ coronary disease

Oxidative stress induced by superoxide (O\textsubscript{2}\textsuperscript{-}) is considered an important factor in the development of atherosclerosis and coronary artery disease. The mechanisms of O\textsubscript{2}\textsuperscript{-} production in nonphagocytic cells are not fully understood; however, it has become clear that NADH/NADPH oxidase plays an important role as the source of O\textsubscript{2}\textsuperscript{-}. Vascular smooth muscle cells (SMCs) lose the ability to produce O\textsubscript{2}\textsuperscript{-} by transfection with the antisense of p22\textsuperscript{phox}, a component of NADH/NADPH oxidase, indicating the essential role of p22\textsuperscript{phox} in O\textsubscript{2}\textsuperscript{-} production.\textsuperscript{1} p22\textsuperscript{phox} is reportedly expressed in nonphagocytic cells such as fibroblasts, endothelial cells, and SMCs.\textsuperscript{1-3} Thus, p22\textsuperscript{phox} is probably a common component in phagocytic and nonphagocytic NADH/NADPH oxidase, and it is essential for the activation of this oxidase system.

Recently, we found that polymorphism of the p22\textsuperscript{phox} gene is associated with coronary risk.\textsuperscript{4} In human coronary arteries, however, the localization of p22\textsuperscript{phox} has never been examined. The aim of this study was to investigate the localization of p22\textsuperscript{phox} and its differences in expression between nonatherosclerotic and atherosclerotic coronary arteries.

Human Tissue

Human coronary arteries were collected from 11 autopsy cases (from persons aged 33 to 86 years). For immunohistochemistry and immunofluorescence examination, serial tissues were embedded in OCT compound and snap-frozen in liquid nitrogen.

Immunohistochemistry

Immunohistochemistry was performed as previously described.\textsuperscript{5} Primary antibodies were rabbit polyclonal anti-human p22\textsuperscript{phox} antibody against the synthetic peptide corresponding to the carboxy-terminal (residues 175 to 194)\textsuperscript{6} and monoclonal antibodies against SM2 and SMemb (Yamasa Corporation). In some experiments, an antibody against the amino-terminal (residue 1 to 25)\textsuperscript{6} of human p22\textsuperscript{phox} was used. For a negative control, the primary antibody was replaced with rabbit serum.

Double-Labeling Immunofluorescence

The antibodies used in double staining were mouse monoclonal anti-human CD68 antibody (DAKO) for macrophages, mouse monoclonal anti-human smooth muscle \(\alpha\)-actin antibody (DAKO) for SMCs, mouse monoclonal anti-human von Willebrand factor antibody (DAKO) for endothelial cells, and mouse monoclonal anti-human prolyl 4-hydroxylase antibody (DAKO) for fibroblasts. TRITC-conjugated anti-rabbit immunoglobulin (DAKO) and FITC-conjugated anti-mouse immunoglobulin (Amersham Pharmacia Bio-

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Western Blotting Analysis

A homogenate of vessels (100 μg of protein) was applied on 15% SDS-polyacrylamide gels. Anti-human p22\textsubscript{phox} antibody and horse-radish peroxidase–labeled donkey anti-rabbit immunoglobulin (Amersham) were used as primary and secondary antibodies, respectively. The signals were detected by the ECL method.

Semiquantitative Analysis of p22\textsubscript{phox} in Immunohistochemistry

The expression of p22\textsubscript{phox} in each segment was graded as follows: grade 0, negative stain; grade 1, variable or weak stain; grade 2, moderately or strongly positive stain. The sections were blindly graded by 3 independent senior pathologists.

Data are expressed as mean±SD. Differences were tested by the Mann Whitney method and considered significant at *P*<0.01.

Results

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All sections were examined by hematoxylin and eosin staining and classified into nonatherosclerotic coronary arteries (without thickening or with only mild and diffuse intimal thickening; 21 segments) and atherosclerotic arteries (47 segments).

In nonatherosclerotic coronary arteries, weakly positive immunoreactivity of p22\textsubscript{phox} was observed mainly in the adventitia. Its expression was scarcely detectable in the endothelium, neointima, or media (Figure 1A, a). The cells expressing p22\textsubscript{phox} in the adventitia were fibroblasts; they were positive for the anti–prolyl 4-hydroxylase antibody.

In atherosclerotic coronary arteries, various histopathological changes were observed, including hypercellular lesions and advanced atheromatous lesions such as fibrous and

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**Figure 1.** A, Immunohistochemical examination of p22\textsubscript{phox} in human coronary arteries. a through c, Nonatherosclerotic coronary artery; d through f, atherosclerotic coronary artery (hypercellular region); g through i, atherosclerotic coronary artery with advanced atheromatous plaques. a, d, and g show hematoxylin and eosin staining. b, e, and h show immunohistochemistry of p22\textsubscript{phox} in the same samples, and c, f, and i show negative controls. LC indicates lipid core (bar=200 μm). B, Immunoblot analysis of p22\textsubscript{phox} in nonatherosclerotic and atherosclerotic coronary arteries. N indicates nonatherosclerotic segments, and A, atherosclerotic segments.
lipid-rich plaques. The immunoreactivity of p22phox was more pronounced in atherosclerotic than nonatherosclerotic arteries. Positive immunoreactivity was detectable through the vessel wall. p22phox was expressed in the adventitia, neointima, media, and endothelium. In hypercellular lesions, its expression was intense in accumulating cells, such as macrophages and SMCs in the neointima (Figure 1A, e). In advanced atheromatous lesions, strongly positive immunoreactivity was detected in neointimal macrophages and some SMCs (Figure 1A, h). Interestingly, intense localized expression of p22phox was observed in macrophages accumulating at the border of atheromatous plaques (the "shoulder region"). Little stain existed, however, in the center of the lipid core. Very similar results were observed using an antibody against the amino-terminal of p22phox (data not shown).

Semiquantitative analysis was performed to compare the expression of p22phox. In the endothelium, neointima, media, and adventitia, p22phox scores in atherosclerotic arteries were significantly higher than in nonatherosclerotic arteries (Table). Western blotting demonstrated that p22phox expression was detected at various levels, but it tended to be more enhanced in atherosclerotic than in nonatherosclerotic segments (Figure 1B).

Characterization of p22phox-Expressing Cells

To identify the types of p22phox-expressing cells, double staining was performed. Most of the p22phox-expressing cells in hypercellular lesions were positive for CD68 (Figure 2A, a through c). Some of the p22phox-expressing cells in atheromatous lesions were positive for α-actin (Figure 2A, d through f). These results suggested that macrophages and some SMCs accumulating in atheromatous lesions might acquire the ability to express p22phox with the progression of atherosclerosis. p22phox-expressing cells in adventitia were positive for a marker of fibroblasts (Figure 2A, g through i), and those in the endothelium were positive for von Willebrand factor (Figure 2A, j through l). For further characterization of p22phox-expressing SMCs, their phenotypes were examined using SM2 and SMemb antibodies. Interestingly, the majority of p22phox-expressing SMCs in atherosclerotic plaques were positive for SMemb but not SM2 (Figure 2B).

Discussion

In the present study, we demonstrated that p22phox, the essential component of NADH/NADPH oxidase, was expressed in human coronary arteries. In nonatherosclerotic coronary arteries, p22phox was expressed mainly in the adventitia. In atherosclerotic arteries, the expression of p22phox protein was enhanced through the vessel wall. Double staining revealed that p22phox-expressing cells were fibroblasts, macrophages, SMCs, and endothelial cells. Thus, adventitial fibroblasts constitutively expressed p22phox, and most macrophages accumulating in atheromatous lesions expressed this component. As atherosclerosis progresses, some SMCs and endothelial cells might acquire the ability to express p22phox. Interestingly, the majority of p22phox-expressing SMCs were positive for SMemb, a maker of undifferentiated SMCs, but not SM2, a maker of differentiated cells. These results suggest that the redox state in the vascular tissue might affect the modulation of cell phenotypes.

Some differences of enzymatic characteristics between phagocytic and nonphagocytic NADH/NADPH oxidases are reported. The nonphagocytic oxidase seems to be constitutively active, and it does not exhibit oxidative bursts, as does the phagocytic oxidase. In contrast to the nonphagocytic oxidase, the NADH-dependent activity in phagocytes is lower than NADPH-dependent activity. However, only limited information is available regarding its molecular structure. The phagocytic oxidase consists of 5 subunits: p22phox, gp91phox, p47phox, p67phox, and rac. The expression of these components in nonphagocytic cells is in contention; however, p22phox is reportedly expressed in endothelial cells, fibroblasts, and SMCs. Rat p22phox cDNA cloned from the SMC library has 81% homology to human neutrophil p22phox, although the human cDNA of nonphagocytic cells has not been cloned. In the present study, nonphagocytic cells were positive for antibodies against the C-terminal and N-terminal of human neutrophil p22phox, indicating that human nonphagocytic p22phox is immunologically identical to phagocytic p22phox. Thus, p22phox may be a common component of phagocytic and nonphagocytic oxidase. Moreover, the functional importance of p22phox in O2- production in nonphagocytic cells is supported by several investigations.

Interestingly, the intensive expression of p22phox was observed in macrophages at the shoulder region, which is the most frequent site of plaque rupture. Circumferential stress was concentrated near the shoulder region, and matrix metalloproteinase (MMP-1), a key enzyme of plaque instability, was overexpressed there. Because reactive oxygen species upregulate MMP, it is interesting to speculate that enhanced expression of p22phox might increase local production of O2-, which in turn, participates in the instability of plaques by upregulating MMP.

In conclusion, the NADH/NADPH oxidase p22phox was expressed in human coronary arteries, and its expression in atherosclerotic arteries was more intense than in nonatherosclerotic arteries. Neointimal and medial SMCs, infiltrating macrophages, adventitial fibroblasts, and endothelial cells in atherosclerotic plaques expressed p22phox. Given the importance of oxidative stress, upregulated p22phox may participate in the process of atherosclerotic coronary disease.

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Figure 2. A, Double-labeling immunofluorescence was performed to identify types of p22phox-expressing cells. Left panels show immunofluorescence of cell-specific markers. Anti-CD68 (a), anti-α-actin (d), anti-prolyl 4-hydroxylase (g), and anti-von Willebrand factor antibodies (j) were used as markers of macrophages, SMCs, fibroblasts, and endothelial cells, respectively (green). Middle panels (b, e, h, and k) show immunofluorescence labeling of p22phox protein (red). Right panels (c, f, i, and l) show double-immunofluorescence. Colocalization of cell-specific markers and p22phox is shown by yellow-labeled immunofluorescence (bar = 20 μm). B, Immunohistochemistry of p22phox (a), SM2 (b), and SMemb (c) in atherosclerotic plaques. The majority of p22phox-expressing SMCs are positive for SMemb but not SM2 (bar = 20 μm).
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