Increased Expression of Oxidative Stress in Proliferated Vascular Smooth Muscle Cells in Early Stage of Human Restenosis After Coronary Angioplasty

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A large body of evidence indicates that oxidative stress has been implicated in the development of restenosis formation after angioplasty in experimental animal models and clinical studies. However, the mechanism by which cells remove oxidative stress as a mediator of restenosis formation in vascular response to balloon-injury remains unclear and data concerning human tissue is lacking.

This study was designed to clarify the role of oxidative stress in the process of neointimal hyperplasia formation of human restenosis lesions after coronary angioplasty. We allocated 122 neointimal hyperplasias obtained by directional coronary atherectomy (DCA) from 115 consecutive patients with restenosis into four groups, according to the time interval until restenosis formation after angioplasty. Cellular and matrix components of neointimal hyperplasia were immunohistologically studied among the groups. To gauge proliferative activity of cellular components, active proliferating cells were identified using antibody to proliferating cell nuclear antigen (PCNA) and positive cells were determined by double-label immunocytochemistry with α -actin, CD-68, leukocyte common antigen, Factor VIII and XIII, respectively. To characterize the potential role of oxidative stress in the process of neointimal hyperplasia, we also assessed the effect of 4-hydroxy-2-nonenal (HNE), a component of oxidatively modified lipids, on cellular components of restenosis.

Consequently, the subject materials for this study consisted of 7 specimens at 0 to 1 month, 42 at 2 to 3 months, 57 at 4 to 6 months and 16 at 7 to 12 months after angioplasty. Neointimal hyperplasia could be histologically categorized into four morphological types of neointimal vascular smooth muscle cells (VSMCs). Predominant granulative type with large round nuclei of VSMCs and inflammatory cells was observed in 4 (57.1%) of the 7 specimens resected at 0 to 1 month after angioplasty. Granulative and mildly edematous type with medium-sized round nuclei of VSMCs and matrix deposits was found in 18 (42.9%) of the 42 specimens resected at 2 to 3 months. Out of 57 specimens resected at 4 to 6 months, markedly myxoedematous type with stellate-shape nuclei of VSMCs and more profound matrix deposits was predominantly found in as many as 39 (68.4%) lesions than that in other phases. In contrast, at 7 to 12 months, finely collagenized type with atrophic nuclei of VSMCs was detected in 10 (62.5%) of 16 specimens. The mean PCNA ($12.6 \pm 4.9\%$) index of VSMCs in the neointima at 0 to 1 month was significantly greater than that at 2 to 3 month ($6.7 \pm 2.1\%$) and 4 to 6 month ($0.4 \pm 0.2\%$) after angioplasty ($p < 0.001$). Moreover, the large nuclei of VSMCs in the predominant granulative type were strongly positive for HNE immunostaining compared with those of stellate VSMCs in the late phase of myxoedematous type neointima (9.3% vs. 0.1%, $p < 0.001$).

In conclusion, these data suggest that oxidative stress could play a pivotal role in proliferation of synthetic VSMCs, which has potential to produce extracellular matrix in development of restenosis early after angioplasty.

Key words : oxidative stress, human restenosis, vascular smooth muscle cell proliferation, extracellular matrix remodeling, atherectomy

Introduction

The repair of vascular injury is a concerted response involving complex signaling pathway that contributes to the clinical feature of post-angio-

plasty restenosis¹⁾. Vascular injury leads to the activation of various biological cells and the release of numerous vasoactive, thrombogenic, and mitogenic factors in the process of restenosis. Restenosis is histologically attributed to neointimal hyperplasia

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(IH) of vascular smooth muscle cells (VSMCs) with luminal constriction due to vascular negative remodeling. VSMCs proliferation and migration from the media to the intima within the arterial wall occurs in restenosis and is associated with a concomitant phenotypic transformation of the VSMC from a contractile to a synthetic state. Therefore, the initiation of the excessive proliferative state in vascular VSMC occurring with phenotype switching under the stimuli of numerous mitogens and growth factors is generally recognized to play a pivotal role in the development of restenosis lesion after vascular injury.

Increased generation of active oxygen species by inflammatory cells and vascular wall cells such as VSMCs and endothelial cells in oxidative stress may potentiate vascular lesion formation²⁾. Reactive oxygen species may participate in growth, migration and apoptosis of VSMCs and thus play a pivotal role in restenosis formation³⁾.

Previous experimental studies^{4)~8)} revealed that various antioxidant drugs reduced not only directly but indirectly the extent of IH after oversized balloon injury in animal model of coronary restenosis, and clinical trials^{9)~16)} also showed that antioxidant inhibits growth of IH and reduces restenosis. Experimental data of mRNA analysis level demonstrated that various active oxygen species significantly stimulated DNA synthesis and c-myc mRNA levels in growth-arrested or proliferating vascular VSMCs to stimulate directly VSMCs proliferation¹⁷⁾.

Oxidative stress is biologically ubiquitous and defined as the imbalanced redox state and may play an important stimulator for VSMC growth after arterial injury. Oxidative stress occurs in cells and tissues when balance of free radical generation and oxidative defense mechanism is altered. The most likely source for free radical production is activation of inflammatory cells. Activated neutrophils and macrophages release superoxide anion and hydrogen peroxide^{18) 19)}. Additionally, endothelial cells and VSMCs can generate active oxygen species and could act in an autocrine fashion to stimulate IH after vascular injury. However, the application of data from currently available animal models to human restenosis mechanism is limited and animal studies may differ considerably from studies of human restenosis lesions. In particular, experimental studies have examined carotid arteries or aorta of

animals, not coronary arteries. On the contrary, in humans, little histological observation is available regarding VSMC activity in restenosis after coronary angioplasty.

The aim of our study was to characterize the histologic and immunohistochemical cellular and matrix interactions of restenosis in the time course after coronary angioplasty, and then to clarify the role of oxidative stress in tissue remodeling at injured vascular wall in human coronary arteries.

Methods

Patients and coronary atherectomy specimens

We obtained 132 specimens of coronary arteries from 115 consecutive patients with restenosis within 12 months after interventional coronary angioplasty. These patients had undergone directional coronary atherectomy (DCA) as previously described²⁰⁾. The formation of IH was observed in 122 (92%) of the 132 specimen between 20 days and 11 months 27 days (130 ± 75 days, mean $\pm$ SD) after initial coronary angioplasty.

The study specimens included 61 specimens of post-balloon angioplasty, 60 specimens of post-directional atherectomy, 1 specimen of post-rotational angioplasty, and then enrolled in this study. The study population consisted of 103 males and 12 females, aged 31 to 83 years, mean age 59.1 ± 9.3 . Since the coronary intervention, none of the patients had received any antioxidant drugs during the medical follow-up until DCA. The study population was allocated into four groups re-catheterized at 0 to 1 month, 2 to 3 months, 4 to 6 months, and 7 to 12 months after the initial intervention. Specimens including IH were evaluated and then compared immune-histologically among the four groups.

Tissue collection and preparation

The restenotic lesions were all resected within 1 to 12 months after therapeutic coronary intervention. Specimens obtained at DCA were immediately immersed and fixed in Carnoy solution for 24 to 48 hours, dehydrated in a graded series of alcohol and xylene, and embedded in paraffin blocks. DCA specimens were stained with Hematoxylin-eosin, Movat's pentachrome, azan-Mallory and alcian blue (pH 2.5) stains, and then examined with light microscopy.

Immunohistochemistry and cell counting

In order to analyze the relationship between proliferation of neointimal VSMCs and oxidative stress, the immunohistochemical double stainings were performed with mouse monoclonal antibodies to proliferative cell nuclear antigen (PCNA), as a marker of ongoing DNA synthesis and cell proliferation, and to 4-hydroxy-2-nonenal (4-HNE), a marker of oxidative stress. To identify specific cell types and their distribution in IH of restenosis, immunohistological stainings for VSMCs (α -actin), macrophages (CD68), lymphocytes (LCA), fibroblasts (Factor XIII-A), and endothelial cells (Factor VIII) were also performed. All of these antibodies were obtained of 1 : 100 following the standard techniques (avidin-biotin-peroxidase, ABC method). Actively proliferating cells were identified using antibody to PCNA. The identity of PCNA-positive cells was determined by double-label immunocytochemical staining, and the proliferative PCNA index (PCNA-positive cells/total cells $\times$ 100) was counted at the representative gridded cross-section.

The number of proliferating VSMCs in the neointima was determined by Immunohistochemical double-staining of serial sections with α -actin monoclonal mouse antibody and PCNA antibody against PCNA (1 : 250 dilution, Dako). We also performed immunocytochemical double-labeling with 4-HNE and VSMC antibodies, CD68, LCA, Factor XIII-A, Factor VIII, respectively. And then we counted the number of PCNA-positive and 4-HNE positive cells which were composed of IH in restenotic lesions per a slide at $\times$ 400 light magnification.

Statistical analysis

Data are expressed as means $\pm$ SE and were analyzed by ANOVA and the Student's t-test or chi-square test for unpaired data. Statistical significance was accepted at $p < 0.05$ level.

Results

IH was found in 122 (92%) of 132 specimens of coronary arterial stenosis lesions resected from 115 consecutive patients within one year after coronary intervention. The atherectomy-derived restenotic specimens consisted of 7 specimens within 1 month, 42 specimens at 2 to 3 months, 57 specimens at 4 to 6 months, and 16 specimens at 7 to 12

months after interventional coronary angioplasty. There was no significant difference among the 4 groups as regard clinical characteristics, risk factors for coronary artery disease and medications (Data not shown).

The neointimal VSMCs hyperplasia of restenosis was histologically categorized into four types according to type of VSMC and extracellular matrix in neointima formation : 1) A predominant granulative type is histologically defined as neointimal hyperplasia composed of VSMCs proliferation with large round nuclei with inflammatory cells infiltration and neovascularization. 2) A granulative and mildly edematous type is also defined neointimal hyperplasia with medium-sized round VSMC nuclei. 3) A markedly myxoedematous type is well recognized as a typical restenotic histological finding with stellate-shaped VSMCs proliferation with a myxoid loose tissue composed of abundant proteoglycan deposits. 4) A finely collagenized type is defined as organizing neointimal hyperplasia with atrophic VSMCs arranged in parallel with collagen fibrous tissue, as shown in Figure-1.

The granulative type of IH is histologically granulation tissue composed of large VSMC nuclei, inflammatory cells and neovascularization and was predominantly observed in 5 (71.4%) of the 7 restenosis tissue resected at 0 to 1 month early after angioplasty (Figure-2). The large VSMCs were strongly positive for PCNA ($12.6 \pm 4.9\%$) and 4-HNE ($9.3 \pm 1.1\%$) staining (Figure-3 and 4). In this early phase, the extracellular component was edema but no accumulation of any matrix components. On the contrary, in 39 (68.2%) of the 57 specimen resected at 4 to 6 months after angioplasty, the main cellular components of IH were stellate-shaped VSMCs which haphazardly arranged with abundant extracellular matrix. The stellate-shaped VSMCs were negative for either PCNA ($0.4 \pm 0.2\%$) or 4-HNE ($0.1 \pm 0.2\%$) staining. Little or no PCNA-positive cells including VSMCs, macrophage and endothelial cell was observed in the neointima at 4 to 6 months later after angioplasty. The major component of the extracellular matrix deposition in the later phase of restenosis was proteoglycans, which was confirmed by strong positive staining with alcian blue staining that disappeared with hyaluronidase digestion. The extracellular matrix (ECM) accumulation produced by neointimal VSMCs was

Figure-1 Histological classification of neointimal hyperplasia according to time course after balloon angioplasty.

Histological features of neointimal hyperplasia at 0-1 month, 2-3 months, 4-6 months and 7-12 months after coronary angioplasty. Within one month early after angioplasty, granulative type of neointimal hyperplasia is seen in. (A) both immature, dedifferentiated SMCs with large nuclei (arrows) and CD-68 positive macrophages (data not shown) in the neointima show strongly PCNA positivity compared with (B) and (C) in the late phase of neointima formation. On the contrary, between 4 to 6 months after angioplasty, stellate-shaped SMCs produces abundantly extracellular matrix (proteoglycans) which is strongly positive with alcian blue staining. And finally, 7 to 12 months, SMCs are atrophic and parallel in increased collagen tissues instead of proteoglycans.

Figure-2 Percentage of subtypes of neointimal hyperplasia according to the duration of restenosis formation after interventional coronary angioplasty.

negligible at 0 to 3 months, but dramatically increased between 4 to 6 months after angioplasty, and then ECM depositions decreased and were entirely replaced with collagen tissue at 7 to 12 months after angioplasty (Figure-1). At 7 to 12 months, the neointimal VSMCs were atrophied,

shrunk, and regularly arranged in collagen connective tissue.

Discussion

It is widely recognized that, immediately after endothelial denudation by balloon-wall injury in arteries, extensive death of medial SMCs occurs with rupture of internal elastic lamina, which then is followed by significant proliferation and migration of the remaining medial SMCs²¹. Numerous experimental data have demonstrated that DNA synthesis in neointimal SMCs peaks at 48 hours after vascular injury, whereas the total accumulation of SMCs in the neointima reaches a maximum at 2 weeks in injured carotid arteries of animals. Proliferation of neointimal SMCs continues for up to 12 weeks with no discernible increase in SMC number. The proper and ecological growth-control of the rapid, extensive medial SMCs viability after injury

Figure-3 Histological assessment of DCA-derived restenotic tissue early after coronary angioplasty is shown by Hematoxylin and eosin (H&E) stain, alcian blue stain with single label immunocytochemistry with α -smooth muscle cell (SMC) actin, and double immunolabeling for 4-HNE and α -SMC actin. (A) Restenotic neointima formation attached to old hypocellular fibrous plaque is seen with H&E stain. Hypercellularity of neointimal hyperplasia is especially noted with background of myxoedematous matrix accumulation. (B) Abundant extracellular matrix, high grade of proteoglycans content (blue-green), is seen with alcian blue stain. (original magnification, 40 $\times$). (C) Double immunolabeling for 4-HNE and α -SMC actin, and then the square area of neointima is magnified. Arrow indicates immature, dedifferentiated SMCs on the left as indicated by a pink nucleus with light brown cytoplasm. And arrowhead indicates CD 68-positive macrophage on the left as indicated by a pink bare nucleus (original magnification, 200 $\times$).

has been experimentally documented to be mainly regulated by apoptotic cell death²²⁾. Apoptosis of proliferated VSMCs, which usually occurs from 7 to 30 days after vascular injury, regulates the number of proliferating SMCs in the neointima at late time^{22)~24)}. The balance of proliferation and apoptosis during the healing response and aberrations of this process may be important factors in lesion progression of restenosis formation as well as atherosclerosis. However, little concerning the mechanism of tissue remodeling in the process of restenosis in humans is known.

Our findings propose four time phases with different morphologic stages of neointimal hyperplasia formation as tissue repair process after vascular injury. The early reparative phase is characterized by predominant granulative type of neointimal proliferative VSMCs with inflammatory cells and neovascularization. Our human data of proliferative activity index of PCNA-positive VSMCs is maximal in less than one month after angioplasty. This find-

Figure-4 Semiquantitative analysis of PCNA-positive and/or 4-HNE positive SMCs in neointimal hyperplasia is shown according to the period after angioplasty. The number of PCNA-positive, 4-HNE positive SMCs is significantly increased within one month after vascular injury. No or little PCNA as well as 4-HNE positive SMC is detected more than 4 months.

ing is consistent with the results of previous animal model experiments of restenosis^{21)~26)}. Experimental animal study of PCNA-positive VSMCs index in 0-28 days following balloon injury showed that, after vascular injury, VSMCs proliferation peaked at 3 days in the tunica media and at 7 days in neointima and increased progressively up to 21 days until endothelial re-growth^{21) 26)}. In the second proliferative phase, phenotype of proliferated VSMCs is shifted and transformation morphologically from large round nuclei to spindle-shape VSMCs is induced. The synthetic phenotype allows the cells to rapidly proliferate and produce extracellular matrix products. Therefore, in the third phase, profound accumulation of extracellular matrix deposits secreted by increased numbers of SMCs leads to a volume gain of neointimal hyperplasia, and causes a clinical restenosis event. The main component of extracellular matrix glycosaminoglycan in restenosis is proteoglycan composed of hyaluronan and versican complex, which has a pivotal role in many critical events, including cell migration, proliferation, differentiation, tissue remodeling, development, and controls vessel wall thickening in cardiovascular disease^{27)~29)}. Neointimal SMCs are precisely responsible for hyaluronan matrix deposition after vascular injury, and hyaluronan itself can modulate SMCs motility and dedifferentiation. Hyaluronan forms a highly molecular weight hydrophobic complex that trap large amounts of water and cause

tissue swelling. The rapid expansion of restenotic lesions in the late phase after angioplasty could be due, in part, to the swelling pressure brought on by the accumulation of the loose myxoid extracellular matrix. Therefore, the regulation of hyaluronan synthesis might provide the finetuning of tissue remodeling after vascular injury to avoid restenosis. Finally, a shift to contractile phenotype with organized ECM, dense fibrous tissue, ultimately provides a structural blood vessel wall in the late phase. A homeostasis between cell proliferation and apoptosis seemed to be reached, possibly resulting in a growth retardation of the neointima in restenosis in later phase after balloon-vascular injury. Although we did not examine to clarify the presence of apoptotic VSMCs in the neointimal formation, atrophy or loss of proliferated SMCs by program cell death as apoptosis in development of neointima could be a possible explanation for the transformation of a stellate SMCs rich-neointimal hyperplasia with abundant proteoglycan matrix accumulation towards a fibrous, cell-poor intimal thickening.

A large body of information on the features of vascular restenotic tissue after balloon injury has been obtained from experimental animal models. However, an enormous gap might exist between histological data of animal models and in humans. Different from animal material, human samples could be obtained only from postmortem^{30)~35)} and from endarterectomy^{36)~39)}. Previous human postmortem studies^{30)~35)} after coronary balloon angioplasty or stenting have morphologically provided neointimal tissue responses at the vascular injured site and revealed the distribution and location of neointimal formation in the whole vessel wall at the injury site. However, it is a truism that the studies of autopsied cases were naturally limited in the number and incidental observations in time course after therapeutic angioplasty. Therefore, fragment sparse data are available by autopsy studies in humans regarding the time-dependent process of neointimal tissue remodeling in restenosis. The present study provided accurate and sequential observations of tissue remodeling, particularly in early phase of human restenosis after angioplasty and, to the best of our knowledge, our observation of four morphologic stages of neointimal tissue remodeling in human has not previously described.

Oxidative stress has been implicated in several steps leading to the development of vascular disease^{41) 42)}. ROS are generated in vascular cells by NAD (P) H oxidases, uncoupled endothelial nitric oxidase (NO) synthase, and other enzymatic sources, or as a byproduct of mitochondrial respiration^{2) 41)}. In balloon-injury artery, ROS are initially derived from invading macrophages and oxidative lipid modification is involved in the vessel wall. Subsequently, ROS are produced by all vascular cells such as injured endothelial cells, activated inflammatory cells, medial smooth muscle cells and adventitial fibroblasts⁴³⁾ and ROS-mediated crosstalk may exist among the endothelium, adventitia and medial SMC layers⁴⁴⁾. We demonstrated that a lipid peroxidation product is detected in proliferated SMCs with large nuclei in neointima within a few months after angioplasty. We used a specific antibody directed against 4-HNE adducts to examine the expression of oxidative stress in neointimal hyperplasia. Lipid peroxidation leads to the generation of a variety of products such as 4-HNE and malondialdehyde that originates exclusively from phospholipid-bound arachidonic acid. Therefore, 4-HNE is thought to be the most reliable index of free radical induced lipid peroxidation as a marker of oxidative stress in tissues⁴⁵⁾. The present study showed that 4-HNE antigenicity was strongly detected in both large SMCs and medium SMCs in neointimal hyperplasia. No studies have demonstrated the immunohistochemical localization of oxidative species products in human restenotic lesions except in the restenosis model study of Ruef *et al.*⁴⁶⁾. They showed that the presence of 4-HNE-adducts was greatly increased in a pattern that is largely extended with the presence of increased vascular endothelial growth factor (VEGF) in the endothelium and neointima 7 days after vascular injury. They have also examined that the increased expression of VEGF mRNA and protein was markedly induced by H₂O₂ in vascular SMCs in balloon-injured arteries. Therefore, they suggested that reactive oxygen species might play a role in neovascularization and SMCs proliferation of injured vessels by regulating VEGF expression and sequential endothelial cell proliferation. HNE is generally found to be toxic to cells at high concentration, while lower concentrations induce DNA synthesis and cell growth^{45)~50)}. HNE causes a con-

centration-dependent increase in intracellular ROS production. Watanabe *et al.*⁴⁹⁾ demonstrated by using explant method of growth-arrested vascular SMCs of rabbit aortic wall *in vitro* that HNE stimulates SMCs proliferation in a concentration-dependent manner and acts synergistically with 5-hydroxytryptamine, as a mitogen for SMCs, in inducing SMCs proliferation. It is suspected that HNE at low concentration regulates growth factors such as platelet-derived growth factor to mediate DNA synthesis, whereas at high concentration HNE causes apoptosis in vascular SMCs with releasing cytokines including tumor necrosis factor⁵⁰⁾. This bifunctional role of HNE may be relevant in atherosclerosis and neointimal formation, where cell growth, apoptosis, and necrosis with tissue remodeling are thought to contribute mutually to lesion formation and progression. Experimental study⁴⁸⁾ using mitochondrial function-deficient vascular SMCs has demonstrated that intracellular ROS generation induced by HNE was not enhanced in mitochondrial function-deficient VSMCs compared with control cells. It is plausible that restenosis may reflect failure in apoptotic pathways, via imbalanced redox state, mediating regression of neointimal hyperplasia after vascular injury. Oxidative stress might play a permissive or indirect role in the process at some points early in the development of neointimal hyperplasia after vascular injury.

Several limitations of this study should be considered. First, no comparisons were made with coronary specimens obtained from patients receiving antioxidant drugs. Second, we did not have any information or data concerning whether or not the study population had taken any antioxidant foods. Finally, we could not fully evaluate the composition and structure of the whole vessel wall at injured site, because the atherectomy specimen does not allow one to examine the entire plaque tissue including tunica media and adventitia.

Conclusions

We found that proliferating immature smooth muscle cells with large nuclei, which are strongly positive for both PCNA as well as HNE stainings, are abundant in neointimal hyperplasia at 0 and 1 month with a decrease by 4 months after coronary balloon angioplasty in humans. The present study

indicate that the direct roles of oxidative stress might involve mainly cell proliferation or early apoptosis rather than events such as productive accumulation of extracellular matrix deposits in the late phase of vascular repair process. Our findings suggest that endogenous oxidative stress is critically involved in early vascular signaling, contributing to vascular smooth muscle cell proliferation after angioplasty-induced vascular injury. Therefore, it is suggested that appropriately concentrated antioxidant coverage immediately and early after vascular injury may be essential. Further studies are necessary in order to clarify the mechanism whether and how early redox processes may affect late phase of vascular repair reaction arising from injury.

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