A Nifedipine-Sensitive Smooth Muscle Cell Population Is Present in the Atherosclerotic Rabbit Aorta

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We evaluated the ability of the Ca\textsuperscript{2+} channel blocker nifedipine to influence the severity of atherosclerotic lesions and the pattern of aortic smooth muscle cell (SMC) differentiation in cholesterol-fed New Zealand White rabbits. The animals were fed a 1% cholesterol-enriched diet for 12 weeks. After 4 weeks of the diet, some rabbits were given nifedipine (20 mg b.i.d.) for another 8 weeks without discontinuation of the cholesterol-enriched diet (experiment 1). Another group of rabbits was treated with nifedipine from the beginning of the cholesterol-enriched diet for the entire 12 weeks (experiment 2). The severity of atherosclerotic lesions was determined by computerized planimetry, and qualitative effects of nifedipine on SMCs were studied by monoclonal antibodies specific for smooth muscle and nonmuscle myosins. In the aortic media of normal rabbits, these antibodies can identify an SMC population with an "immature" type of myosin pattern; a marked increase in the number of these cells is observed during atherogenesis. In experiment 1, we observed a marked decrease of medial SMCs with the immature type of myosin pattern, without any significant reduction in atherosclerosis severity. In experiment 2, disappearance of the previously mentioned medial SMC population was accompanied by a dramatic slowing of intimal lesion development. These results indicate that nifedipine treatment is effective in reducing atherosclerotic lesions only when given from the beginning of a cholesterol-enriched diet. Delay of nifedipine administration until the fourth week of the cholesterol-enriched diet fails to halt progression of the disease. The observed antiatherosclerotic activity can be attributable to a direct effect of the drug on the medial SMC population, which increases during the course of experimental atherogenesis. (Arteriosclerosis and Thrombosis 1991;11:928-939)

In the last decade a number of studies have been published dealing with the potential direct antiatherogenic effect of Ca\textsuperscript{2+} channel blockers, mainly nifedipine\textsuperscript{4-10} and verapamil\textsuperscript{11,12}. In most of these studies Ca\textsuperscript{2+} channel blockers were revealed to be effective in reducing the development of atherosclerotic lesions in hypercholesterolemic rabbits.\textsuperscript{4,6} Nifedipine was also able to induce the regression of atherosclerotic lesions in cholesterol-fed rabbits in which the diet was discontinued at the same time the drug was administered.\textsuperscript{5} In addition, Ca\textsuperscript{2+} channel blockers were reported to be effective in reducing myointimal proliferation after balloon injury.\textsuperscript{10} Finally, a significant slowing of the appearance of new coronary lesions was demonstrated by angiography in patients with mild to moderate coronary heart disease who were treated with nifedipine.\textsuperscript{13,14}

The mechanisms responsible for the antiatherogenic effects of Ca\textsuperscript{2+} channel blockers are not fully understood.\textsuperscript{1,11,15} Studies performed in vivo and in vitro suggest that two main mechanisms underlie the observed effects of these drugs on atherogenesis: 1) alteration of lipid metabolism in the arterial wall\textsuperscript{16-19} and 2) inhibition of the proliferative activity and migration of vascular smooth muscle cells (SMCs).\textsuperscript{20,21}

In a previous study,\textsuperscript{22} we have shown the existence of a medial SMC population with an "immature" type of myosin expression, which markedly increases in size during experimental atherogenesis. This unique medial SMC population closely resembles the major cell population present in the atherosclerotic plaque. It is reasonable to assume that if this medial
SMC population plays a role in the formation of the atherosclerotic lesions, then the inhibition of its growth could be beneficial in preventing the development of atherosclerosis.

The present study has been undertaken to 1) confirm the ability of nifedipine to promote the regression (experiment 1) and/or to prevent the development (experiment 2) of atherosclerotic lesions in cholesterol-fed rabbits and 2) evaluate the effect of nifedipine on the differentiation pattern of vascular SMCs in the atherosclerotic rabbit aorta and in primary cell cultures.

**Methods**

**Study Design**

Forty-nine male New Zealand White rabbits weighing about 2.6 kg were obtained from a stable breeding stock and maintained on standard rabbit chow for several days. Then 36 animals were fed a cholesterol-enriched diet containing 1% cholesterol and 8% corn oil for 12 weeks and were divided into four groups: 1) group 1 (eight rabbits) was killed after 4 weeks of cholesterol feeding; 2) group 2 (eight rabbits) was killed after 12 weeks of cholesterol feeding; 3) group 3 (10 rabbits) was force fed nifedipine slow-release tablets (20 mg b.i.d., Bayer A.G., Leverkusen, FRG) from the fourth to the 12th week of the cholesterol-enriched diet (experiment 1); and 4) group 4 (10 rabbits) was force fed nifedipine slow-release tablets (20 mg b.i.d.) from the first to the 12th week of the cholesterol-enriched diet (experiment 2). Five age-matched rabbits fed the standard diet were used as controls. Another eight rabbits fed the standard diet were given nifedipine slow-release tablets (20 mg b.i.d.) for 12 weeks. At the fourth, eighth, and 12th weeks from the beginning of the cholesterol-enriched or control diet, body weight and blood pressure were measured. At the same time, blood samples were collected for standard biochemical analysis and serum cholesterol and triglyceride levels. The nifedipine serum levels were determined by gas chromatography in blood samples taken 1 hour before drug administration.

At the end of the study period the animals were killed while under anesthesia with phenobarbital, and the aortas were immediately removed. The entire thoracic aorta except for a standard ring taken near the aortic valve, which was used for immunocytochemical studies, was fixed under a constant pressure of 90 mm Hg in 10% buffered formalin for 15 minutes. The specimens for immunocytochemistry were immediately frozen under liquid N2 and stored at -70°C until use. In two of 10 animals from groups 3 and 4, the entire aorta including the bifurcation was removed and examined for immunocytochemistry.

**Computerized Morphometry of Rabbit Aortas**

The formalin-fixed specimens of aortas from normal, hypercholesterolemic, and hypercholesterolemic nifedipine-treated rabbits were embedded in paraffin, and serial histological sections (1 μm thick) were taken at 5-mm intervals. A total of nine sections per aorta were stained with Weigert's-van Gieson's stain and analyzed by computerized planimetry. Sections were projected onto a graphic tablet (Calcomp 2000, Digitizer Products Division, Anaheim, Calif.) with a Zeiss microscope (Oberkochen, FRG) coupled with "camera lucida" equipment. The contours of the medial and intimal layers were traced on the graphic tablet, which was connected to an M24 personal computer (Olivetti, Ivrea, Italy), and the areas of both layers were calculated for each section. In each animal group the mean values of the areas of the medial and intimal layers were determined. The data were analyzed by one-way analysis of variance.

**Tissue Cultures**

Medial SMCs were obtained from the thoracic aortas of adult New Zealand White rabbits by enzymatic dispersion as previously described. The presence of contaminating endothelial cells was excluded by staining with anti-von Willebrand factor antibodies (Behringwerke A.G., Marburg, FRG). The cells were seeded in 30-mm plastic wells in Dulbecco's modified eagle's medium (Flow Laboratories, Irvine, UK) without serum. Seeding density was 4 x 10⁴ cells/well. Twenty-four hours after seeding, nifedipine was added to the culture medium at a final concentration of 10⁻⁵ M and was replaced every 24 hours for 5 days. To avoid binding of nifedipine to plasma proteins, a synthetic serum (CPSR 1, Sigma Chemical Co., St. Louis, Mo.) was added to the culture medium. Control cultures were grown under the same conditions without addition of nifedipine to the culture medium. Subconfluent cultures of both nifedipine-treated and control cells were fixed in acetone and processed for immunofluorescence as described below.

**Immunocytochemistry**

Cryosections (4 or 10 μm thick) of aortic tissue from the different animal groups were processed for indirect and double immunofluorescence and direct immunoperoxidase according to the procedures in use in our laboratory. Two monoclonal anti-myosin antibodies were used in this study. SM-E7 is an antibody specific for an antigenic epitope shared by myosin heavy-chain 1 and heavy-chain 2 isoforms of smooth muscle type present in rabbit aortic smooth muscle. "NM-G2 is an antibody raised against human platelet activomyosin and directed against a myosin heavy-chain epitope present in non-muscle cell types." Appropriate dilutions of anti-myosin antibodies in 1% bovine serum albumin in phosphate-buffered saline (PBS) were applied to cryosections and incubated for 30 minutes at 37°C in a humidified chamber. Sections were subsequently rinsed in PBS, and bound antibodies were revealed by rabbit anti-mouse immunoglobulin G (IgG) coupled with fluorescein (FITC) or rhodamine (RITC) isothiocyanate (Dako, Dakopatts a/s, Glostrup, Denmark). Controls included sections stained with 1) the...
fluorescent antibody alone and 2) mouse nonimmune IgG followed by the fluorescent antibody. Specimens were examined with a Zeiss Axioplan microscope equipped with an HBO 100-W high-pressure mercury light and selective FITC filter (Nos. 447765 and 447738) and Neofluar x 10 (numerical aperture 0.30), x20 (numerical aperture 0.50), x40 (numerical aperture 1.40) objective lenses. Photomicrographs were taken with Kodak Technical Pan Film (Eastman Kodak Co., Rochester, N.Y.) and developed with Kodak HC fluorescent antibody alone and 2) mouse nonimmune IgG followed by the fluorescent antibody. Controls included sections stained with 1) the secondary antibody. Specimens were examined with the SM-E7 antibody coupled with FITC and NM-G2 indirectly labeled with RITC according to the procedure previously described. Bound anti-mycos antibodies were also revealed by a rabbit anti-mouse IgG conjugated with horseradish peroxidase (Dako) with 3,3’-diaminobenzidine and H2O2 as substrates. Endogenous peroxidases were removed by CH3OH/H2O2 treatment before application of the antibodies. Controls included sections stained with 1) the secondary antibody alone and 2) mouse nonimmune IgG followed by the secondary antibody. Specimens were examined with an inverted Zeiss IM 35 microscope and Zeiss Neofluar x 10 (numerical aperture 0.25) or x16 (numerical aperture 0.40) objective lenses. Photomicrographs were taken, and the films were developed as described above.

Results

Biochemical and Morphometric Studies

Throughout the study period, no significant difference was found in blood pressure and body weight among the various groups of rabbits. Serum cholesterol and triglyceride levels are shown in Table 1. Both of these parameters were not modified after nifedipine treatment. No appreciable difference was detected among the various groups of animals in terms of the biochemical parameters of renal and hepatic function, glucose, and Ca2+ serum levels.

All nifedipine-treated rabbits showed nifedipine serum levels in the range of 33.8±49.8 ng/ml at the beginning of treatment and 21.6±25.0 ng/ml at the end of the study period. These values are close to those found in humans during treatment with the usual therapeutic dosage of nifedipine (20 mg b.i.d.).

Table 2 shows the results of the morphometric analysis performed on aortas from normal, hypercholesterolemic, and hypercholesterolemic nifedipine-treated rabbits. No significant difference was found in the cross-sectional area of the medial layers among the various animal groups examined. Conversely, a marked increase in the area of the intima, as measured from transverse sections, was observed among the various groups of animals in terms of the biochemical parameters of renal and hepatic function, glucose, and Ca2+ serum levels.

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FIGURE 1. Photomicrographs of indirect immunofluorescence assay of normal (panel A) and atherosclerotic (panels B–D) rabbit aortas. Aortas from hypercholesterolemic animals after 4 (panel B), 8 (panel C), and 12 (panel D) weeks of cholesterol feeding were reacted with NM-G2 antibody. A few medial NM-G2-positive cells (asterisks) are present in the normal aorta (panel A), whereas they increase in number after cholesterol feeding. These cells are located mainly in the medial region (m) beneath the internal elastic lamina (iel). e, Endothelium; ap, atherosclerotic plaque. Scale bars in panels A, B, and C are 60 μm and in panel D, 50 μm.

measured in normocholesterolemic untreated animals (data not shown).

Immunocytochemistry

Two SMC populations have been previously identified in the media of normal and atherosclerotic rabbit aortas by use of the NM-G2 anti-myosin antibody.22

Figure 1 shows the immunoreactivity of the cells present in the intima and underlying media of rabbit aortas with the NM-G2 antibody after 4, 8, and 12 weeks of cholesterol feeding. Almost all the cells in the thickened intima are labeled by this antibody (Figures 1B–1D); a progressive increase in intimal thickness is also observed from the fourth to the 12th week of cholesterol feeding. In the aortas of normocholesterolemic rabbits the intima is not thickened, and NM-G2 antibody recognizes only the endothelium and a few scattered medial cells (Figures 1A and 3A). These latter medial cells are doubly labeled with NM-G2 and SM-E7 antibodies (data not shown, see Reference 22). The size of this SMC population markedly increases during atherogenesis22 (Figures 1B–1D); at 8 weeks of cholesterol feeding, NM-G2-positive cells are located mainly in the medial region just beneath the internal elastic lamina (Figure 1C).

Figures 2 and 3 show the immunocytochemical patterns observed in the aortas of hypercholesterolemic nifedipine-treated rabbits in experiment 1 (i.e., nifedipine treatment beginning after 4 weeks of cholesterol feeding). After nifedipine treatment, the cells present in the atherosclerotic plaque show an immunofluorescence pattern with SM-E7 and NM-G2 antibodies (Figures 2A–2D), which is very similar to that observed in hypercholesterolemic untreated rabbits; that is, the majority of cells are labeled with both antibodies, whereas a minority of cells are labeled with either SM-E7 or NM-G2 (not
FIGURE 2. Photomicrographs of indirect (panels A and B) and double (panels C–F) immunofluorescence tests of atherosclerotic nifedipine-treated rabbit aortas (experiment 1). Aortas from hypercholesterolemic animals treated with nifedipine from the fourth to the 12th week of cholesterol feeding were reacted with SM-E7 (panels A, C, and E) or NM-G2 (panels B, D, and F) anti-myosin antibody. SM-E7 antibody stains all medial smooth muscle cells and part of the cells present in the plaque (panels A and C). Conversely, NM-G2 antibody is uniformly reactive with the atherosclerotic plaque (panel B); very rare smooth muscle cells are stained by this antibody in some regions of the underlying media (panel D, asterisks). These latter cells are doubly stained with both SM-E7 and NM-G2 antibodies (panels E and F, dotted lines). Note that in the plaque the majority of cells are reactive with both antibodies (panels C and D). ap, Atherosclerotic plaque; iel, internal elastic lamina; m, medial layer. Scale bars in panels A–D are 60 μm and in panels E and F, 20 μm.
FIGURE 3. Photomicrographs of indirect immunoperoxidase analysis of normal (panel A), cholesterol-fed (panel B), and cholesterol-fed nifedipine-treated (panel C, experiment 1) rabbit aortas with NM-G2 anti-nonmuscle myosin antibody. In normal aortas, rare medial cells are recognized by NM-G2 antibody (panel A, asterisks). In the media underlying the atherosclerotic plaque (panel B), NM-G2-positive cells (asterisks) are increased and localized mainly beneath the internal elastic lamina (iel, arrowhead). In aortas from hypercholesterolemic animals treated with nifedipine (panel C), these medial cells are absent. Note that the atherosclerotic plaque in panels B and C shows the same staining pattern with NM-G2. e, Endothelium; m, medial layer; ap, atherosclerotic plaque. Scale bars in panels A and B are 40 μm, and in panel C, 60 μm.
two antibodies are localized in the same cytoplasmic filaments (Figures 6A and 6B). However, in a few cells the distribution of smooth muscle and nonmuscle myosin immunoreactivities follows two different patterns (Figures 6C and 6D; see also Reference 22). Very rare cells are reactive with SM-E7 (Figures 6A and 6B) or NM-G2 (Figures 6E and 6F) alone.

In SMCs cultured in the presence of nifedipine a different pattern of immunostaining was observed. Almost all cells are labeled by SM-E7 alone (Figures
FIGURE 5. Photomicrographs of indirect immunofluorescence assay of aortas of normocholesterolemic rabbits treated with nifedipine for 12 weeks. Aortic sections were reacted with SM-E7 (panel A) and NM-G2 (panel B) anti-myosin antibodies. No NM-G2-positive cells were detected in the medial layer (m) after nifedipine treatment (panel B), whereas cells reactive with SM-E7 antibody were still present (panel A). e, Arrowhead, endothelium. Scale bar in panels A and B is 50 μm.

Discussion

Contrasting results have been reported in the literature about the potential antiatherogenic effects of nifedipine, as measured by the extent of atherosclerotic lesions and/or cholesterol accumulation in the aortic wall. These discrepancies may be attributable to the different animal models used in these studies, cholesterol-enriched diet regimens, drug dosages, bioavailability of nifedipine, and routes of administration of the drug.

In agreement with other authors, we have found that nifedipine is very effective in preventing the development of atherosclerotic lesions, whereas it has no effect on preexisting lesions. A possible explanation of this differential effect of nifedipine can be obtained from the immunocytochemical studies performed with normal and atherosclerotic rabbit aortas (Reference 22 and this study).

The distinct differentiation pattern shown by vascular SMCs during atherogenesis and the development of intimal thickening has been interpreted as being due to 1) a process of “phenotypic modulation” of the fully differentiated medial SMCs, 2) a “dedifferentiation” process, and 3) recruitment and proliferation of medial “stem-like” cells. It has been demonstrated that the expression of smooth muscle-specific cytocontractile and cytoskeletal protein markers such as vinculin, caldesmon, and actin, characteristic of differentiated SMCs, is markedly reduced in the intima of human atherosclerotic aortas. A similar protein switching process was reported for desmin, vimentin, and actin isoforms in the proliferation of SMCs that follows the endothelial denudation induced by balloon injury. Using a 90-kd surface antigen as a marker of SMC differentiation, Ju Printseva et al obtained evidence for fetal type SMCs in the human atherosclerotic plaque. As previously discussed, myosin isoforms in vascular smooth muscle can be conveniently used as differentiation markers to study vascular myogenesis and the pathological processes in which SMC proliferation is involved. Using this approach, we have been able to demonstrate that the aortic media of normal adult rabbits contains an SMC population that shows the presence of both smooth muscle and nonmuscle myosin isoforms. During atherogenesis in hypercholesterolemic rabbits this immature medial SMC population markedly increases in size, and a similar cell population appears in the atherosclerotic plaque. The immature medial SMC population, which might represent the main source of intimal cells found in the plaque, virtually disappears when rabbits are treated with nifedipine from the beginning of cholesterol feeding (experiment 2, Figure 4). A marked decrease in the size of this medial SMC population is also observed in experiment 1. However, this change is not accompanied by a significant reduction in intimal lesions compared with those of untreated cholesterol-fed rabbits. The different effects of nifedipine on both intima and media of the atherosclerotic rabbit aorta in experiment 1 could be accounted for by differences in nifedipine sensitivity between the atherosclerotic plaque and the underlying media. This different sensitivity can be related to distinct functional properties of the SMCs in these two vascular layers. In fact, SMCs isolated from rabbit atherosclerotic lesions and grown in vitro display a higher proliferation rate than do those obtained from the normal media. Different levels of platelet-derived growth factor (PDGF)–like activity were demonstrated in the intima and media of the
FIGURE 6. Photomicrographs of double immunofluorescence on primary cultures of smooth muscle cells obtained from normal rabbit aortas and grown in the absence of nifedipine. Acetone-fixed cells were treated with SM-E7 antibody directly labeled with fluorescein isothiocyanate (panels A, C, and E) and NM-G2 antibody indirectly stained with immunoglobulin G (IgG) anti-mouse IgG coupled with rhodamine isothiocyanate (panels B, D, and F). Most of the cultured cells display double reactivity with both anti-myosin antibodies (panels A and B, filled star). A minority of SMCs, however, are labeled with SM-E7 (panels A and B, asterisk) or NM-G2 exclusively (panels E and F, open star). Cells with double reactivity to SM-E7 and NM-G2 antibodies occasionally show a different intracellular distribution of myosin immunoreactivity (panels C and D, arrowheads). Scale bar for panels A–F is 8 μm.
normal arterial wall^36 and in SMCs isolated from the intimal layer after endothelial denudation.\textsuperscript{37,38} On the other hand, we cannot rule out the possibility that nifedipine concentration within the atherosclerotic lesions is not high enough to induce any appreciable quantitative and/or qualitative effect at this level. From these observations, we can argue that nifedipine is effective as an antiatherogenic drug only when it is administered in the early phase of atherogenesis and of medial SMC "activation." This hypothesis is in agreement with the data obtained by Jackson et al\textsuperscript{10} on SMC proliferation after balloon injury of rabbit aortas. These authors reported that nifedipine was highly effective in preventing myointimal SMC proliferation when the drug was administered within 48 hours after the injury; in contrast, no effect was detected when nifedipine treatment was delayed by 7 days. Moreover, Nilsson et al\textsuperscript{20} showed a significant reduction in tritiated thymidine incorporation by cultured vascular SMCs only when nifedipine was added to the culture medium within 15 minutes after stimulation with PDGF. The results of the present study indicate a possible mechanism by which nifedipine prevents the development of atherosclerotic lesions. It is still unclear whether the observed nifedipine-induced changes in myosin isoform composition in vascular SMCs are causally related to the reduced severity of atherosclerotic lesions. Other possible mechanisms have been postulated to explain the antiatherogenic effect of nifedipine, including 1) inhibition of SMC proliferation\textsuperscript{20}; 2) inhibition of SMC migration\textsuperscript{21}; 3) stimulation of cholesteryl ester in lipid-laden SMCs\textsuperscript{39}; and 4) changes in macrophage cholesterol homeostasis.\textsuperscript{16-19}

It is reasonable to assume that the antiatherogenic effect of this drug on medial SMCs was achieved by decreasing the intracellular Ca\textsuperscript{2+} concentration in arterial SMCs.\textsuperscript{11,15} At the present time we cannot say whether nifedipine acts as a blocker of the proliferation of putative medial stem-like cell activation and/or as an inhibitor of phenotypic modulation of medial SMCs. It is known that the Ca\textsuperscript{2+} ionophore A23187 can alter the expression of myosin light-chain isoforms in cultured skeletal muscle cells.\textsuperscript{40} According to some authors,\textsuperscript{41} the cytoplasmic Ca\textsuperscript{2+} availability has a specific role in the maintenance of a stable level of myosin synthesis. A direct effect of nifedipine on the differentiation pattern of vascular SMCs is
suggested by the results of our tissue-culture experiments. The absence of reactivity of nifedipine-treated cultured SMCs with NM-G2 antibody could be in concordance with 1) the instability of nonmuscle myosin isoform in the myofilaments and 2) the inhibition of the synthesis of the nonmuscle myosin isoform and thus the induction of the differentiated SMC phenotype. The observed changes in the immunocytochemical pattern of medial SMCs with NM-G2 antibody are unlikely to be due to conformation variations in myosin. In fact, unfixed cryosections of rabbit aortas treated with ionic (sodium dodecyl sulfate) and neutral (Triton X-100, Nonidet NP-40, and Tween-20) detergents for 10 minutes at 37°C maintain the same staining pattern with NM-G2 antibody (not shown).

In conclusion, our study indicates that nifedipine treatment is effective in reducing atherosclerotic lesions only when given from the beginning of a cholesterol-enriched diet. Delay of drug administration until the fourth week of the cholesterol-enriched diet fails to halt progression of the disease. The observed antiatherogenic effect of nifedipine is accompanied by a dramatic change in a medial SMC population that increases during the course of experimental atherogenesis.

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References


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