Calcium Antagonists Differently Inhibit Proliferation of Human Coronary Smooth Muscle Cells in Response to Pulsatile Stretch and Platelet-Derived Growth Factor

Zhihong Yang, MD; Georg Noll, MD; Thomas F. Lüscher, MD

**Background.** Vascular smooth muscle cell proliferation is the key event of coronary artery disease. Mechanical forces, in particular, pulsatile stretch and platelet-derived growth factor, may play an important role.

**Methods and Results.** Vascular smooth muscle cells were cultured from the media of human left descending coronary arteries obtained from organ donors using the explant method. To study effects of pulsatile stretch on vascular smooth muscle cell proliferation, a computer-controlled in vitro pulsatile stretch device was used. Cells were seeded onto Flex I culture plates with deformable membranes and exposed to pulsatile stretch (60 cycles per minute) and/or growth factors. Proliferation of smooth muscle cells was determined by \(^{3}H\)-thymidine incorporation. Pulsatile stretch markedly stimulated \(^{3}H\)-thymidine incorporation of coronary smooth muscle cells (180±15 to 432±27 cpm/10^4 cells; \(P<.05\)) after 24 hours and increased cell number after 6 days (10.3±0.7×10^4/mL to 11.7±0.5×10^4/mL; \(P<.05\)). Platelet-derived growth factor–AB (0.01 to 10 ng/mL) concentration-dependently stimulated \(^{3}H\)-thymidine incorporation in coronary smooth muscle cells (EC_{50}, 0.1 ng/mL) and had additive effects with pulsatile stretch. The Ca^{2+} antagonist verapamil (10^{-7} to 10^{-5} M) concentration-dependently inhibited proliferation stimulated by platelet-derived growth factor back to control levels (\(P<.05\) to .01) but not that induced by pulsatile stretch.

**Conclusions.** Pulsatile stretch and platelet-derived growth factor are potent stimuli for proliferation of coronary smooth muscle cells. The selective inhibitory effect of a Ca^{2+} antagonist on smooth muscle cell proliferation stimulated by platelet-derived growth factor but not by pulsatile stretch may explain why the drugs have only modest antiatherogenic effects in patients with coronary artery disease. (Circulation. 1993;88:832-836.)

**Key Words** • cells • muscle, smooth • growth factors • calcium • brief communication

Coronary artery disease is an important cause of morbidity and mortality.1 The major event in coronary artery disease is an obstruction and/or occlusion of one or more epicardial coronary arteries,2,3 leading to myocardial ischemia and eventually infarction, arrhythmias, and death. The three most important factors for coronary artery obstruction are (1) increased vasoconstrictor responses,4-6 (2) decreased antithrombotic properties of the coronary blood vessel wall,7-9 and (3) proliferation and migration of vascular smooth muscle cells.10

Although certain cardiovascular drugs reduce coronary vasoconstriction11,12 and thrombus formation,7,8,13,14 it remains uncertain whether some of the currently used drugs have antiproliferative properties in humans.15-17 Effective prevention and/or reversal of proliferative changes in coronary artery disease requires a better understanding of the stimuli involved. These may include mechanical forces18,19 as well as growth factors released from platelets, endothelial cells, leukocytes, and/or vascular smooth muscle cells.10 As not all experimental animals develop coronary atherosclerosis, species differences may be relevant. Therefore, in this study, human coronary artery smooth muscle cells in culture were used, and proliferative responses to pulsatile stretch and platelet-derived growth factor–AB were studied. In addition, the effects of the Ca^{2+} antagonist verapamil on proliferative responses to pulsatile stretch and platelet-derived growth factor–AB were investigated.

**Materials**

Bovine serum albumin was from Fluka (Switzerland), recombinant platelet-derived growth factor–AB was purchased from Boehringer Mannheim (Mannheim, FRG), verapamil was from Sigma (St Louis, Mo), and all tissue culture materials were from Gibco (Basel, Switzerland). \(^{3}H\)-Thymidine was from Amersham (Zürich, Switzerland), trichloroacetic acid was from Fluka, and perchloric acid was from Merck, Switzerland; monoclonal antibody against α-smooth muscle actin was from Sigma.
Cell Isolation and Cultivation

Vascular smooth muscle cells were obtained from human coronary arteries from organ donors. The cells were isolated by a modified explant method. The media of left descending coronary arteries was isolated under a microscope, cut into 1-mm² pieces, and placed onto plastic tissue culture plates coated with 0.1% gelatin in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20 mM L-glutamine and HEPES buffer solution, 100 U/mL penicillin, and 100 μg/mL streptomycin, containing 20% fetal calf serum in a humidified atmosphere (37°C; 95% air–5% CO₂). The medium was replaced every 3 days. Cells were passaged by trypsinization (0.05%). Experiments were performed between passages 5 and 10. Smooth muscle cells were characterized by an indirect immunofluorescence immunohistological staining using specific anti-smooth muscle α-actin antibodies.

In Vitro Pulsatile Stretch Device for Cultured Cells

To study effects of pulsatile stretch on human vascular smooth muscle cell proliferation, an in vitro pulsatile stretch device was used (Flexcell Corp, McKeesport, Pa). Cells were seeded onto Flex I culture plates coated with type-1 collagen at an initial density of 10⁶/mL. After 24 hours of incubation to allow for cell attachment, culture media were replaced with serum-free medium (DMEM) containing 0.2% bovine serum albumin (instead of 20% fetal calf serum) and all other ingredients given above for 48 hours to obtain quiescent nondoning cells. Then, the serum-free medium was removed and replaced by 0.5% fetal calf serum culture medium; Flex I culture plates then were placed on a computerized Flexercell Strain Unit gasketed baseplate in the incubator and subjected to cyclic mechanical stretch (60 cycles per minute; 25% elongation at the periphery of the culture plate bottom). In parallel, other Flex I culture plates not subjected to pulsatile stretch served as controls. Under the same experimental conditions, certain cells were exposed to various substances and drugs (platelet-derived growth factor–AB, the calcium antagonist verapamil) that were used to study putative mechanisms of smooth muscle cell proliferation. Verapamil was added immediately before the cells were exposed to platelet-derived growth factor or pulsatile stretch and was present throughout the entire experiment.

Studies With Platelet-Derived Growth Factor–AB

In another series of experiments, the effects of platelet-derived growth factor–AB on smooth muscle cell growth were studied. The cells were seeded onto 12-well plates at a density of 5 x 10⁴ per well and treated as described above except that the cells were exposed to platelet-derived growth factor–AB (instead of pulsatile stretch) in either the presence or the absence of the calcium antagonist verapamil (10⁻⁷ to 10⁻⁵ M).

Assay of Cell Mitogenicity

To study cell growth, DNA synthesis as assayed by ³H-thymidine incorporation and cell number was measured. After 24 hours of pulsatile stretch and/or stimulation with platelet-derived growth factor–AB, cells were pulsed with ³H-thymidine (1 μCi/mL; 70 to 85 Ci/mol; Amersham) for 4 hours and then washed with phosphate-buffered saline and ice-cold 10% trichloroacetic acid (30 minutes at 4°C); the incorporated radioactivity was measured with a β-counter (LKB Wallac, MBV AG, Switzerland). For study with pulsatile stretch, cell number was determined on the second and sixth days of stretch with an electronic counter (Coulter Electronics).

Statistical Analysis

All experiments were performed in triplicate, unless indicated. Data are presented as mean±SEM. ANOVA followed by Scheffe’s test for repeated measurements was used for statistical analysis. A two-tailed P<.05 was considered to indicate a statistical difference.

Results

Pulsatile Stretch

After 24 hours of pulsatile stretch, ³H-thymidine incorporation was more than doubled in coronary artery smooth muscle cells (control, 180±15 cpm/10⁶ cells; pulsatile stretch, 432±27 cpm/10⁶ cells; n=2 independent experiments in triplicate; P<.05). At this point, cell number was not yet changed, but after 6 days of stretch, cell number was significantly increased (control, 10.3±0.7 x 10⁴/µL; pulsatile stretch, 11.7±0.5 x 10⁴/µL; P<.05).

Platelet-Derived Growth Factor

Platelet-derived growth factor–AB (0.01 to 10 ng/mL) concentration-dependently stimulated ³H-thymidine incorporation after 24 hours in coronary smooth muscle cells (n=3 independent experiments in triplicate). The half-maximal effects occurred at about 0.1 ng/mL (80% increase of control), whereas the maximal effect was reached at 1 ng/mL (244% increase of control).

Interaction of Pulsatile Stretch and Platelet-Derived Growth Factor

In human coronary artery smooth muscle cells, pulsatile stretch (1617±100 cpm/10⁶ cells; control: 717±18 cpm/10⁶ cells; P<.05) and platelet-derived growth factor–AB (0.1 ng/mL; 1147±73 cpm/10⁶ cells; P<.05 vs control) alone stimulated ³H-thymidine incorporation (Fig 1). When platelet-derived growth factor and pulsatile stretch were applied together, the stimuli had additive effects on ³H-thymidine incorporation of coronary smooth muscle cells (Fig 1; 2409±122 cpm/10⁶ cells; P<.05 vs platelet-derived growth factor–AB or stretch control).

Effects of Ca²⁺ Antagonist

After 24 hours of stimulation, ³H-thymidine incorporation of human coronary artery smooth muscle cells in response to pulsatile stretch was not inhibited by the Ca²⁺ antagonist verapamil (10⁻⁷ to 10⁻³ M; Fig 2, left). In contrast, verapamil (10⁻⁴ to 10⁻⁶ M) markedly inhibited platelet-derived growth factor–AB (0.5 ng/mL) stimulated ³H-thymidine incorporation in the SMC (IC₅₀: <10⁻⁷ M, Fig 2, right).

Discussion

This study demonstrates that mechanical forces, such as pulsatile stretch and a growth factor like platelet-
The degree and extent of pulsatile stretch depend on the level of blood pressure, particularly systolic blood pressure, and the frequency of the stimulus (ie, heart rate). Blood pressure and heart rate are independent predictors of cardiovascular morbidity and mortality. \(^1\) \(\beta\)-Blockers, which reduce both the extent and frequency of pulsatility, reduce such events after myocardial infarction. \(^2\) This study for the first time demonstrates that in cultured human coronary smooth muscle cells, rhythmic stretch induces proliferative responses, in terms of both \(^3\)H-thymidine incorporation and cell number. Similar results were obtained in human vascular smooth muscle cells obtained from the saphenous vein, \(^2\) whereas in porcine vascular smooth muscle cells from aorta the response appears to differ. \(^2\) The difference between our results and those of the latter study may be related to the different protocol as in the latter much longer cycles of stretching and relaxation (three stretch cycles per minute) were used, whereas we have applied 60 stretch cycles per minute to mimic average heart rate in patients. The latter authors also used a higher cell density and performed experiments with nonquiescent cells, since the cell number and \(^3\)H-thymidine incorporation increased dramatically under control conditions. \(^2\) As in human coronary artery smooth muscle cells, proliferative responses to pulsatile stretch also

in culture. The \(\Ca\) antagonist verapamil inhibited proliferative responses to platelet-derived growth factor–AB but not those to pulsatile stretch, indicating that they activate different intracellular signal transduction pathways.

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occur in hypertensive rat smooth muscle cells, mesangial cells, endothelial cells, and cardiac nonmyocyte cells.\textsuperscript{27-31} Although responses in vitro and in vivo are difficult to compare, the results of this study in human coronary artery smooth muscle cells are compatible with the concept that mechanical forces are an important determinant in the pathogenesis of coronary artery disease. Pulsatile stretch, however, does not appear to be uniformly effective as a proliferative stimulus in all blood vessels as it was inefficient in cultured smooth muscle cells obtained from the human internal mammary artery.\textsuperscript{23} Interestingly, the mammary artery has a low incidence of atherosclerosis,\textsuperscript{32} which may in part be related to a lack of proliferation in response to mechanical forces. The fact that atherosclerotic lesions only develop within decades suggests that there are important antiproliferative mechanisms (eg, endothelium-derived heparin, heparan sulfates, or transforming growth factor-\(\beta_3\)-\(\beta_3\)), which counteract the proliferative effects of pulsatile stretch in the coronary circulation, particularly in younger patients. It is possible that cardiovascular risk factors and age diminish these inhibitory mechanisms and unmask the proliferative effects of pulsatility and other mechanical forces.

Platelet activation is a major event in coronary artery disease.\textsuperscript{7,8} The fact that inhibition of platelet function exerts a primary and secondary protective effect\textsuperscript{7,8,13} suggests that they are primarily involved in the pathogenesis of coronary artery disease. Indeed, human platelets contain large amounts of platelet-derived growth factor–AB\textsuperscript{24} and transforming growth factor–\(\beta_3\) (albeit in its inactive form\textsuperscript{24}). As studied in several other cell types,\textsuperscript{16,26-36} this study demonstrates that platelet-derived growth factor–AB is a potent mitogen in human coronary artery smooth muscle cells. Indeed, \(3\text{H}\)-thymidine incorporation was stimulated with platelet-derived growth factor–AB in a low concentration range (ie, 0.05 to 1 ng/mL), which may be clinically relevant. The effects of pulsatile stretch and platelet-derived growth factor–AB were additive, suggesting that they have an independent role as mediators of proliferation responses in the human coronary artery.

Numerous pharmacological approaches have been tested to prevent coronary artery disease. As drugs reducing vasoconstriction\textsuperscript{11} and thrombus formation\textsuperscript{13,14} are available, pharmacological tools to inhibit proliferative responses would be of great clinical importance. The results of this study demonstrate that verapamil inhibits proliferative responses induced by platelet-derived growth factor–AB in human coronary smooth muscle cells. As the half-maximal effect already occurred at less than 10\textsuperscript{-7} M, this observation may be of clinically relevance as plasma levels of verapamil in patients reach 4 \times 10\textsuperscript{-7} M.\textsuperscript{39} In contrast, stretch-induced proliferative responses of coronary smooth muscle cells were unaffected. As similar results were obtained with nifedipine (data not shown), this indicates that Ca\textsuperscript{2+} antagonists as a drug class are unable to interfere with that proliferative stimulus. This specific antiproliferative effects of verapamil may also explain why in clinical studies, Ca\textsuperscript{2+} antagonists reduce somewhat but do not prevent new coronary lesions, whereas existing lesions are unaffected.\textsuperscript{17} It is possible that the maintained capacity of pulsatility to stimulate proliferation even in the presence of Ca\textsuperscript{2+} antagonists contributes to the maintenance of existing lesions and reduces the potential inhibitory effects of the drugs on newly developing lesions.

In summary, human coronary smooth muscle cells can be held in culture and used to study proliferative responses to various stimuli and antiproliferative properties of drugs. Mechanical forces and platelet-derived growth factor–AB are important proliferative stimuli for coronary artery smooth muscle cells. The differential effects of Ca\textsuperscript{2+} antagonists on smooth muscle cell proliferation stimulated by platelet-derived growth factor and pulsatile stretch may explain why the drug has some effect but no striking effect on atherosclerotic lesions in patients with coronary artery disease.

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**References**

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