Intimal Injury In Vivo Activates Vascular Smooth Muscle Cell Migration and Explant Outgrowth In Vitro

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In vivo induced endothelial injury results in an intimal thickening, mainly caused by stimulation of smooth muscle cells (SMC), which migrate into, and proliferate in, the denuded intima. We evaluated the manifestation and persistence of this stimulus in several in vitro explant and growth assays using aortic explants from either balloon catheter-injured rats or from sham-operated controls. SMC outgrowth from control explants started 48 hours after explantation, became half-maximal after 96 hours, with 74% of the explants showing outgrowth. Explants from balloon-injured aortas showed SMC outgrowth within 24 hours, became half-maximal after 48 hours, with 92% of the explants showing outgrowth. An interval of 4 to 7 days between balloon injury and sacrifice was optimal for obtaining accelerated outgrowth, while a 1-day interval showed a weak stimulation and a 21-day interval had no such effect. The in vivo stimulated cells grew more rapidly, resulting in a greater colony size per explant, and they became temporarily serum-independent. Increased growth rate persisted up to the second subculture but returned to normal in subsequent passages. Mechanisms of SMC migration can now be quantitatively assessed by replacing the intima with the tissue culture dish, allowing studies on the extent and persistence of atherogenic stimuli. (Arteriosclerosis 4:183–188, May/June 1984)

Migration and proliferation of arterial smooth muscle cells (SMC) into the intima are considered important events in the development of atherosclerosis. Altered endothelial integrity is thought to expose the underlying SMC to stimuli that induce migration of the SMC through the internal elastic lamina to the intima, where they proliferate. Approximately 3 to 4 days after experimental injury, activated or modulated SMC appear at the luminal surface and form an intimal thickening that displays many features of an atherosclerotic plaque.

Cell culture techniques are now widely used to study the growth kinetics and morphology of SMC. While SMC initially collected by enzymatic digestion do not proliferate in vitro and remain contractile for a short period of time, SMC cultured from explants of vascular media produce actively growing, noncontractile cells after a lag period of several days. These proliferating SMC synthesize extracellular matrix materials, such as collagen and glycosaminoglycans; their growth is stimulated by several factors such as those found in platelets and in hyperlipemic, diabetic, and hypertensive sera.

Our working hypothesis is that the readiness of SMC to migrate onto a culture dish and to proliferate in vitro is a measurement of the extent of pathological stimulation that these cells have received in vivo. Indeed, an increase in cell proliferation, as well as morphological and biochemical changes, have been demonstrated in SMC cultured from atherosclerotic rabbits and from hypertensive or diabetic rats. Differences in SMC outgrowth after mechanical injury of the rabbit aorta have also been reported briefly. We have extended this work in the studies reported here by quantifying SMC migration and proliferation in explant cultures and subcultivations after balloon injury of the rat aorta. These studies show that SMC migration and proliferation in vitro are accelerated and enhanced in response to the intimal injury. Furthermore, the in vivo stimulus renders the SMC temporarily serum-independent.

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Methods

**Balloon Injury of the Rat Aorta**

Aortic balloon catheterization was performed on male Wistar rats weighing 300 to 325 g (Charles River Breeding Laboratories, Incorporated, Wilmington, Massachusetts) according to a modification of the method originally developed by Baumgartner. After ether anesthesia, a segment of the left carotid artery was exposed, the distal portion was occluded, and an incision was made for insertion of a No. 2 French Fogarty embolectomy catheter (Edward Laboratory, Santa Ana, California). The catheter was passed into the thoracic aorta to a point below the diaphragm, after which the balloon was inflated with distilled water and brought back to the point of incision. The balloon was passed through the aorta in this manner five times, with a 45° rotation after each passage. After the last passage, the balloon was withdrawn and the carotid artery ligated. Sham-operated animals were used as controls in all experiments; the balloon was placed in the carotid artery but not pushed down into the aorta. The rats were sacrificed at 1, 4, 7, or 21 days after the surgery. Additional controls were performed comparing sham-operated animals with untreated ones or with rats that had been balloon-injured after sacrifice.

**Cell Culture**

The thoracic aortas of injured and control animals were dissected under sterile conditions after the animals were sacrificed. The adventitia was removed, and the intimal side was scraped gently with a scalpel to remove any endothelium. Explants measuring 1 mm × 1 mm were prepared according to the method of Ross and placed with the luminal side down in separate wells in tissue culture, multiwell dishes. Two hours later, after the explants were attached to the surface, 1 ml of medium was carefully added. Dulbecco's minimal essential medium (DMEM) was used supplemented with 10% fetal calf serum. Low-serum and serum-free experiments were performed using DMEM with 0.1% fetal calf serum (FCS) or plain DMEM. The dishes were placed in a 37°C incubator with an atmosphere of 95% air and 5% CO₂.

The percentage of total explants showing smooth muscle cell outgrowth was recorded daily over a period of 8 days. On the 8th day, the explants were removed and the medium changed. The medium was subsequently changed twice a week. Three weeks after the first outgrowth had occurred, the cells were trypsinized, and the number of cells per well were counted with a hemocytometer. Growth curves of pooled cells were obtained in the second subculture by plating cells at a density of 2 × 10⁴ cells/cm² and counting the cells daily, starting 24 hours after plating. Data were analyzed by analysis of variance followed by Scheffe's test (different intervals between surgery and sacrifice) or by Student's t test. The results are expressed as mean values ± the standard error of the mean (SEM).

**Electron Microscopy**

Cells and explants on culture dishes were fixed in situ with 1% glutaraldehyde and 4% formalin for 1 hour, postfixed with 1% OsO₄ for 1 hour, stained with 1% uranyl acetate for 30 minutes, and infiltrated with Epon 812 directly in the culture flask. Ultrathin sections were cut on a Reichert Om U2 ultramicrotome with a diamond knife, placed on 200 mesh copper grids, stained with uranyl acetate and lead citrate, and examined with a Phillips 300 electron microscope.

**Results**

The SMC outgrowth of standardized aortic medial explants from sham-operated control animals began on the second day after explantation in 2.3% of the explants. Half-maximal outgrowth was reached within 96 hours after the explantation. After 8 days, a maximum of 73.7% of the explants showed outgrowth (Figure 1). In the rats that received balloon injury 4 days before sacrifice, SMC outgrowth started as early as 24 hours after explantation and required less than 48 hours to reach half-maximal outgrowth; a maximum of 92.3% of the explants showed outgrowth after 8 days. The difference between the two groups was statistically significant (Figure 1).

To determine the time of optimal stimulation of SMC colony formation, we varied the interval between the balloon injury and the sacrifice of the animals. As shown in Figure 2, the explant outgrowth from animals sacrificed 4 and 7 days after balloon injury was virtually the same. Explants from animals sacrificed after 21 days showed a significantly lower outgrowth for the first 4 days. The outgrowth of explants from animals sacrificed after 1 day was lower than the 4- and 7-day groups for the first 2 days. All subsequent experiments were therefore performed using rats sacrificed 4 days after balloon injury.
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Figure 2. Percentage of colony forming explants from rat aortas sacrificed 1, 4, 7, and 21 days following balloon injury (10% fetal calf serum). (○ = 1 day, ◊ = 4 days, ▲ = 7 days, ▼ = 21 days). The 4- and 7-day groups were significantly higher than the 21-day group on the first 4 days after explanation (p < 0.01) and higher than the 1-day group on the first 2 days after explantation (p < 0.01). Data represent mean values ± SEM of at least five rats per group (18 to 24 explants analyzed per rat).

After migrating out from the explant, the cells started to proliferate, forming multilayered colonies around the explant. The colony size of the explant cultures was determined 3 weeks after outgrowth had started. While control explants averaged $5.9 \times 10^4$ cells per explant, the cell harvest from explants of balloon-injured aortas was approximately twice as much ($11.8 \times 10^4$ cells; p < 0.01).

The growth difference between SMC obtained from control and balloon-injured aortas persisted into the second subculture at a significant level (Figure 3), while fourth-passage cells showed no significant difference between the two groups. No differences were found between explants from sham-operated or untreated rats, or from rats balloon-injured after sacrifice.

We also compared the serum requirements necessary to induce cellular migration and proliferation in vitro. When the control explants were kept under low serum conditions (0.1% fetal calf serum, Figure 4), the earliest outgrowth took place after 5 days, and only 12.6% of the explants showed any cell outgrowth at all. Under the same low serum conditions, explants from the balloon-injured group behaved similarly to those in 10% serum, with initial outgrowth beginning after 24 hours and 83% producing colo-

Figure 3. Cell population growth curve for smooth muscle cells cultured from control (•) and balloon-injured (▲) rat aortas. Second passage cells were seeded with an initial cell density of $2 \times 10^5$ cells/cm² and counted at the specified intermediate time points, and expressed as cell number $\times 10^4$ per 35 mm dish. Data represent mean values ± SEM of six experiments done in duplicate (p < 0.01).

Figure 4. Percentage of colony forming explants from control (●) and balloon-injured (▲) rat aortas in low serum conditions (0.1% fetal calf serum). Data represent mean values ± SEM of six rats per group (18 to 24 explants per rat, p < 0.01 for Days 2 to 8).

Figure 5. Percentage of colony forming explants from control (●) and balloon-injured (▲) rat aortas in serum-free medium. Data represent mean values ± SEM of six rats per group (18 to 24 explants per rat, p < 0.05 for Days 3 and 4, p < 0.1 for Days 5 to 8).
nies. In the total absence of serum, 41.3% of the explants from balloon-injured rats still showed cellular outgrowth after 8 days, compared to 1.4% in the control group (Figure 5). Under these conditions, the temporarily serum-independent cells became quiescent after one to three cell divisions; growth could be reactivated by adding medium containing 10% FCS.

The morphological investigation of the SMC migrating out of the explants revealed typically elongated cells (Figure 6) that started proliferating, actively forming multilayered meshworks in a characteristic hill and valley pattern.

Ultrastructural investigation of the cultured SMC revealed highly active cells with an accumulation of mitochondria, free ribosomes, and a widened rough endoplasmic reticulum (Figure 7) in all groups.

Discussion

Migration of SMC is considered one of the early and essential cellular activities in atherogenesis; in response to a number of stimuli, these cells leave their usual anatomical position and appear in a layer of the vessel wall where conditions for proliferation and synthetic activity are different. From electron micrographs taken at different times after balloon injury, a sequence of events has been suggested consisting of cellular migration followed by proliferation and intense synthesis of extracellular matrix material resulting in a lesion resembling an atherosclerotic plaque.

Our approach of in vivo stimulation by balloon injury and in vitro analysis of the effects allows the isolation, visualization, and quantification of selected mechanisms under controlled conditions. After maximal stimulation of the vessel wall by balloon injury, SMC outgrowth appeared earlier, and more medial explants showed SMC outgrowth compared to explants from control rat aortas. This increased and accelerated outgrowth allows quantification of the part of the balloon injury that stimulates migration.

Our data also indicate that those SMC leaving the media in response to balloon injury behave differently under tissue culture conditions. One of these differences is that the cells grow more rapidly, resulting in a higher cell number per explant in a given amount of time after first cell outgrowth. This might be due to a higher cell number of SMC migrating out of the explant. Alternative explanations for the more rapid growth could be that the balloon injury could select...
Figure 7. Cultured primary smooth muscle cell from a balloon-injured rat aorta showing perinuclear accumulation of mitochondria, free ribosomes, and a widened rough endoplasmic reticulum. There are virtually no filaments at that level. (Bar = 1 μ.)

for a preexistent subpopulation of SMC with a potential of faster growth, or an originally homogeneous population of quiescent cells could have been uniformly stimulated to grow faster. Although increased growth rate persists up to the second subculture, growth rates returned to normal in subsequent passages, favoring the hypothesis of a reversible alteration of quiescent cells rather than a selection of preexisting cells with inherent potential for fast growth.

Balloon catheterization completely removes vascular endothelium, allowing direct access of a variety of humoral and cellular stimulatory factors to the SMC. Balloon catheterization completely removes vascular endothelium, allowing direct access of a variety of humoral and cellular stimulatory factors to the SMC. The temporary serum independence might be an indication that platelet-derived growth factor (PDGF) was bound to the "balloon-injured" SMC in vivo, thereby accounting for the absence of a requirement for exogenous PDGF from the serum in vitro. Several observations suggest that the balloon also inflicts direct injury to the rest of the vessel wall. In vivo the intimal SMC proliferation is self-limiting, despite the persistent absence of endothelium in large areas in the rabbit and in smaller areas in the rat aorta. Similarly, the alteration of SMC that we measured in vitro were most evident in cells from explants taken 4 to 7 days after balloon injury. The SMC alteration decreased in explant cultures started at a later time after injury (21 days) despite the fact that at this time the reendothelialization was not completed.

Whatever the combination of injuries inflicted by the balloon might be, this trauma probably represents a maximum stimulus for the SMC alterations and thus serves as a model for the comparable clinical interactions such as balloon-assisted devices, angioplasty, or grafting. However, unknown stimuli occurring spontaneously in diabetes, hypertension, and hypercholesteremia result in qualitatively similar SMC alteration. For example, SMC cultured from diabetic or hypertensive rats and from hypercholesteremic rabbits showed increased proliferation in vitro in addition to altered prostaglandin production, collagen, and glycosaminoglycan synthesis. Primary SMC cultures from human fatty streaks, but not from fully developed plaques, also showed an increased thymidine uptake. Such ob-
servations support the claim that measurements of in vitro migration and proliferation allow conclusions regarding the extent (but not the nature) of the stimuli received in vivo under atherogenic conditions.

These experiments, as well as earlier balloon experiments, both show that mechanical trauma is used as a maximal stimulus for SMC to migrate and proliferate. The experiments reported here differ, however, in that the intima, where SMC accumulate in vivo, has been replaced with the tissue culture dish; thus, we can assess the effects of the stimulus in a quantitative fashion. We postulate that the readiness of SMC to migrate onto a culture dish and to proliferate, even temporarily without serum, is a measure of the extent of pathologic stimulation that the cells have received.

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