Balloon Catheter Injury to Rabbit Carotid Artery
I. Changes in Smooth Muscle Phenotype

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Stereology was used to investigate the changes in ultrastructure of smooth muscle cells during the formation of an experimental intimal thickening induced by injury with an inflated balloon catheter. The volume density of myofilaments in the cell cytoplasm was measured in smooth muscle cell-lined areas (which are freely permeable to Evans blue dye and, hence, stain blue) and in re-endothelialized areas (which remain white after injection of Evans blue) of the rabbit carotid artery. Two weeks after injury, the volume densities of myofilaments in the intimal smooth muscle cells in both white and blue areas were significantly less than that for control medial smooth muscle (67.8%±3.6%; mean±SE), being 38.8%±1.0% and 35.9±3.3%, respectively. By 6 weeks after injury, the volume density had increased significantly in both white (55.1%±3.4%) and blue areas (53.5%±3.0%), and these values did not change significantly by 18 weeks. The volume density of myofilaments in the luminal (lining) smooth muscle cells in the blue areas was significantly less than that of control medial cells and remained low (26.7%±2.1%) up to 18 weeks after Injury. The initial balloon-induced injury caused considerable damage to the smooth muscle cells in the media, and the remaining medial cells underwent similar changes in ultrastructure to the cells in the adventitia. At 2 weeks, the cells had a low volume density of myofilaments (44.9%±2.4%), which returned to a level not significantly different from the control artery by 6 weeks after injury. There were no differences in the estimates of the volume density of myofilaments between the inner and outer media of the injured arteries. These findings suggest that, after injury produced by a balloon catheter, the smooth muscle cells in both the media and the resultant intimal thickening undergo a reversible change in ultrastructure.

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A characteristic feature of human arteries, such as the coronary artery or the aorta, is a diffuse thickening of the intima (DIT). The DIT develops with age and, by the end of the second decade, this layer may be three times the thickness of the media. Furthermore, it is within the DIT that atheromatous plaques usually develop. Previous stereological studies in our laboratory have shown that most of the smooth muscle cells in areas of DIT adjacent to atherosclerotic lesions exhibit a modified ultrastructure, as indicated by the high volume density of synthetic organelles and the low volume density of myofilaments in their cytoplasm. These cells are morphologically similar to the cells in vitro termed "synthetic" phenotype, which contain large amounts of rough endoplasmic reticulum, free ribosomes, and mitochondria but which have little contractile apparatus. In contrast, in areas of DIT from atherosclerosis-free carotid arteries, the smooth muscle cells exhibit features similar to the "contractile" cells of the underlying media, including a high volume density of myofilaments (V, myo). In vitro, cells of the synthetic and contractile phenotypes have different metabolic characteristics, including responsiveness to mitogens, ability to synthesize extracellular matrix, and uptake of lipoproteins and accumulation of lipid.

Experimental methods of endothelial denudation produce a fibromuscular intimal thickening similar in morphology to the human DIT. The formation of such an intimal thickening involves migration of smooth muscle cells from the media into the intima where some of these cells subsequently proliferate. Previous biochemical and immunofluorescence studies have noted changes in the cytoskeleton of the smooth muscle cells during the development of an intimal thickening.

To determine whether smooth muscle cells in vivo undergo changes in ultrastructure similar to those observed in cell culture, in this study we quantified the changes in the V, myo in the smooth muscle cells of the rabbit carotid artery after balloon catheter injury. These data are important for a subsequent study that examines how, after injury, modifications in artery structure affect the reactivity to various vasoconstrictors.

Methods

A total of 24 male rabbits (2 to 3 kg body weight) from the rabbit colony of the Baker Medical Research Institute, Melbourne, Australia, were used in this study. The animals were maintained throughout the experimental period.
on a restricted diet of 200 g commercial stock pellets per day supplemented with greens.

**Endothelial Denudation**

Endothelial denudation was produced in the common carotid artery by using the balloon catheter model originally developed by Baumgartner. Each animal was preanesthetized with propanidid (i.v., Bayer, Sydney, New South Wales), and anesthesia was maintained with halothane via an endotracheal tube. A midline incision was made in the animal's neck to expose the distal bifurcation of the right common carotid artery. Any small sidebranches were ligated flush to the adventitia of the carotid. Immediately before endothelial denudation, each animal received 500 U of heparin (i.v., Commonwealth Serum Laboratories, Melbourne, Australia). A 2F Fogarty balloon catheter was inserted into the common carotid via a branch of the external carotid artery and was then inflated with air to a level that visibly distended the artery so that the balloon met with resistance during its passage along the artery. The inflated catheter was withdrawn almost to the point of entry, and this whole procedure was carried out a total of three times. A prior pilot study had shown that this was necessary to consistently remove all the endothelium from the experimental segment. After removal of the balloon catheter, the branch of the external carotid artery was ligated, and the skin wound was sutured using 3.0 silk. In each experiment, the unoperated contralateral carotid artery served as a control. At the completion of the operation, each animal was given an intramuscular injection of the antibiotic Reiverin (approximately 40 mg/kg, Hoechst, Australia).

**Fixation and Electron Microscopy**

The experimental animals were killed at intervals between 0 and 7 days and at 2, 6, or 18 weeks for examination by scanning and transmission electron microscopy. One hour before sacrifice, each rabbit received an intravenous injection of Evans blue (60 mg/kg body weight; Chroma Gesellschaft Schmid and Company, Stuttgart, FRG) in 0.9% saline. The animals were overdosed with sodium pentobarbital, and the abdominal aortas were cannulated so that the animals could be perfused in a retrograde manner. Initially, the experimental and control carotid arteries were cleared of blood by perfusion with Hanks' balanced salt solution, pH 7.3. The arteries were then fixed by perfusion for a further 10 minutes with 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). The arteries were then placed in fresh 5% glutaraldehyde for a further 1 to 2 hours before further processing.

After injection of Evans blue, areas of the injured artery lined by modified smooth muscle cells stained intensely blue, while areas with regenerated endothelium retained whiteness. The experimental arteries were sampled at random from areas that stained blue and from areas that remained white, while the control arteries were sampled throughout their length. The tissue was cut into small blocks (1 × 3 mm) of no fixed orientation and processed for scanning and transmission electron microscopy. The tissue was rinsed thoroughly with 0.1 M phosphate buffer (pH 7.3), postfixed for 1 to 3 hours in a 2.5% osmium tetroxide in phosphate buffer, and then rinsed again thoroughly in buffer.

Segments for scanning electron microscopy were dehydrated through graded concentrations of 2-ethoxyethanol and then ethylene glycol before being carefully critical-point dried with liquid carbon dioxide as the transition fluid. Specimens were mounted on metal stubs, were sputter-coated with gold, and were examined at 20 kV in an ETEC Autoscan scanning electron microscope.

Tissue segments for transmission electron microscopy were dehydrated through acetone solutions of graded concentrations and were then embedded in araldite/epon. Thick sections (0.5 to 1.0 μm) were cut and stained with methylene blue for light microscopy. Thin sections were cut with a diamond knife on a Reichert ultramicrotome, mounted on copper mesh grids, stained with uranyl acetate and lead citrate, and then viewed at 80 kV in a Philips 400 electron microscope.

**Stereology**

The phenotype of smooth muscle cells, expressed as the V, myo in the cell cytoplasm, was determined using a modification of the method described by Mosse et al. The V, myo was estimated in cells in the intimal thickening and the media of arteries injured with a balloon catheter. As a control, the V, myo of cells in the media of the unoperated contralateral arteries was also determined.

Thick sections were cut from randomly selected blocks, and thin sections were cut from the first five "suitable" blocks. A suitable experimental block contained an area of intimal thickening in which the plane of section passed from the vessel lumen to the adventitia. Thin sections were mounted on 400-mesh copper grids and were examined at ×4600 magnification.

An unbiased systematic sample of electron micrographs was obtained by photographing the field in the corner of each grid square over which the intimal thickening lay. To determine an appropriate sample size, 10 photographs were taken of cells in the intimal thickening from each of five separate sections of the first experimental artery analyzed. By plotting the cumulative mean V, myo for successive micrographs and finding the number of photographs sampled where the progressive mean stabilized within ±5%, it was found that eight photographs of each section would provide a representative sample. Therefore, the overall sampling strategy used for the remainder of the analysis consisted of eight photographs from five sections of each artery.

For quantification of cells in the intimal thickening, only cells situated between the internal elastic lamina (IEL) and the lining cells were included. Only cells that could be positively identified as smooth muscle cells were included. The morphologic criteria for classification as smooth muscle included in the presence of a basal lamina, plasmalemmal vesicles, and myofilament bundles with associated dense bodies.

In Evans blue-stained areas, the smooth muscle cells that extended to the vessel lumen in each particular section were regarded as luminal cells and were analyzed separately. A systematic sample of micrographs of luminal smooth muscle cells was obtained by photographing
the field where the luminal cell layer crossed each adjacent grid bar. A representative sample size for estimation of the phenotype of the luminal smooth muscle cells determined as described above was also found to be eight photographs from each section. The overall sampling hierarchy for the luminal cells, therefore, consisted of eight photographs from five sections of each artery.

The \( V_m \) of the smooth muscle cells in the media was estimated in Evans blue-stained areas of the experimental arteries. The sample of the media was divided into inner media (smooth muscle cells between the IEL and the third elastic lamellae) and outer media (cells external to the third elastic lamellae). A total of eight photographs was taken of the media by using a similar sampling procedure as for the intimal thickening.

Each negative was enlarged to a final magnification of \( 13 \times 800 \). A double quadratic test lattice having a spacing of 8 and 16 mm and 682 points was placed over the micrographs, and the lattice intersections were used for point counting volumetry. The \( V_m \) in the cytoplasm of smooth muscle cells was determined by counting the number of lattice intersections on the smooth muscle cytoplasm (\( P_{cm} \)) and the number of points over myofilaments (\( P_{mf} \)). For the purpose of this study “myofilaments” were taken to include the entire contractile and cytoskeletal apparatus of the cell, i.e., myosin, actin, and 10 Å filaments, and cytoplasmic- and membrane-associated dense or dark bodies. All other cytoplasmic organelles, inclusions, and surrounding cytosol, including lipid droplets, were taken to be “organelles.” All the test points falling over smooth muscle cytoplasm in any given micrograph were included, thereby allowing the inclusion of small profiles of cells sectioned at a distance from the nucleus. This was a necessary consideration due to the localization of organelles in the perinuclear region of smooth muscle cells. The \( V_m \) was calculated as a percentage of smooth muscle cytoplasm:

\[
V_m = \frac{\sum P_{cm} \times 100}{\sum P_{mf}} \quad (1)
\]

Due to the somewhat irregular distribution of smooth muscle cells in the intimal thickening, the total number of test points falling on each micrograph (and hence in each representative sample) varied considerably. Therefore, the stereological parameter (\( V_m \)) was directly calculated as a ratio of the sum of the point counts over all sections for each animal:

\[
V_m \text{ (for each animal)} = \frac{\sum P_{mf} \text{ for all sections}}{\sum P_{mf} \text{ for all sections}} \times 100 \quad (2)
\]

The overall value of \( V_m \) for each experimental group was then obtained by calculating the mean of the estimates of all the animals.

**Statistical Analysis of Stereological Data**

The estimates of \( V_m \) for the cells of each different category (i.e., intima, luminal cells, inner and outer media, and entire media) were analyzed similarly. The data were tested for homogeneity of variances using Cochran’s C test and were tested for normality by plotting the estimates against their normal probability scores. These tests showed that the data satisfied the requirements for parametric testing. The \( V_m \) estimates for each category were then analyzed using a one-way ANOVA and the Student-Newman-Keuls multiple comparisons test. For all statistical tests, the level of significance was taken to be 5%.

**Results**

In the control rabbit carotid artery, the intima consisted of the lining endothelial cells and a thin layer of subendothelial connective tissue, with very few smooth muscle cells. The \( V_m \) in smooth muscle cells of the media was 67.9±3.6% (mean±SE), indicating that the cell cytoplasm was predominantly occupied by myofilaments (Table 1). The organelles involved with synthesis (i.e., rough endoplasmic reticulum, free ribosomes, and Golgi), as well as mitochondria, were located in the perinuclear region and constituted only a minor portion of the cytoplasmic volume.

Four hours after balloon catheter injury, the entire surface of the injured carotid artery up to within 0.5 cm of the distal bifurcation stained intensely with Evans blue. The endothelium had been completely denuded, with the exception of small patches of cells within and adjacent to the ostia of minute sidebranches (Figure 1). The artery surface was covered by adherent platelets, scattered erythrocytes, and leukocytes. The elastic lamellae remained intact but appeared slightly fragmented, and some necrotic smooth muscle cells were observed throughout the inner two thirds of the media (Figure 2).

**Two Weeks after Balloon Catheter Injury**

Two weeks after balloon catheter injury, endothelial cell regrowth extended 2 to 4 mm from the origin of the carotid artery. This area of regenerated endothelium remained microscopically white after staining with Evans blue, merged sharply with a central region that stained intensely blue, and was lined by luminal smooth muscle cells. In most instances, the blue zone extended 4 to 5 cm in total length but was broken up by variable-sized patches of regenerated endothelium. These patches of endothelium most often occurred toward the distal end of the artery and were associated with ostia of small sidebranches.

A fibromuscular intimal thickening, or neointima, of variable thickness was present in re-endothelialized and smooth muscle-lined areas (see Figure 3). In a few areas, focal eccentric intimal thickenings were also observed. As observed previously, the predominant cell type present was smooth muscle, although occasional mononuclear cells, granulocytes, and other cells not readily categorized were also present. The orientation of the intimal cells seemed to vary between areas. While, in general, most of the intimal smooth muscle cells were aligned longitudinally, variable numbers of cells were orientated obliquely or even concentrically. In several arteries, fibroblasts were observed throughout the neointima, and lipid-containing cells were also observed in a few areas.

In white areas, the regenerated endothelial cells contained abundant synthetic organelles and bordered on neighboring endothelial cells with either simple abut-
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Table 1. Volume Densities of Myofilaments in Smooth Muscle Cells of Media in Arteries after Balloon Catheter Injury

| Artery                        | Inner media | Outer media | Average over entire media |
|-------------------------------|-------------|-------------|---------------------------|
| Control (n=5)                 |             |             |                           |
|                               |             |             | 67.9±3.6                  |
|                               |             |             | (57.9, 77.6)              |
| Evans blue-stained            |             |             |                           |
| Two weeks after injury (n=5)  | 42.7±3.3†   | 46.2±2.3†   | 44.9±2.4*                 |
|                               | (33.5, 51.8)| (39.9, 52.4)| (38.2, 51.5)              |
| Six weeks after injury (n=5)  | 58.5±3.8    | 65.9±1.8    | 62.2±2.5                  |
|                               | (47.9, 69.0)| (60.8, 71.0)| (55.3, 69.2)              |
| Eighteen weeks after injury   |             |             |                           |
| (n=3)                         | 63.9±3.1    | 68.1±1.4    | 65.5±1.7                  |
|                               | (50.4, 77.4)| (59.9, 72.3)| (57.1, 71.7)              |

Each value is the mean volume density of myofilaments expressed as a percentage of the cell cytoplasm ± SE. The numbers in parentheses are the lower and upper limits of the 95% confidence intervals of the means. Inner media includes cells between the internal elastic lamina and the third elastic lamellae. Outer media includes cells external to the third elastic lamellae. n=number of animals.

†Significantly different from the control value (p<0.01) and from 6- and 18-week values for the entire media (p<0.01). 

*Significantly different from the values in the corresponding areas at 6 and 18 weeks (p<0.01). There were no significant differences between the volume densities of myofilaments of cells in the inner and outer media at any time (p>0.05).

Figure 1. Scanning electron micrograph of the rabbit carotid artery 4 hours after balloon catheter injury. The endothelium has been totally denuded except for cells within and adjacent to the ostia of a small sidebranch (arrow). The denuded area is covered by adherent platelets, leukocytes, and red blood cells. Bar=7 μm.

ments or sometimes complex interdigitation (Figure 4). Bundles of microfilaments were observed mainly in the basal cytoplasm. In some regions, these microfilament bundles appeared continuous with filamentous structures in the subendothelial extracellular matrix similar to the fibronexus-like associations observed by Hüttnet et al.25

The smooth muscle cells in the underlying neointima displayed a range of ultrastructure. The majority of cells contained abundant synthetic organelles and only small peripheral bundles of myofilaments (Figure 4). Other cells, however, were observed to contain more moderate amounts of synthetic organelles. The Vₘ, myo (Table 2) in the intimal cells in white areas was 38.8%±1.0%, which
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Figure 2. Transmission electron micrograph of the carotid artery 4 hours after balloon catheter injury. Platelets adhere to the denuded surface. The smooth muscle cells of the media exhibit ultrastructural evidence of degeneration. IEL=intimal elastic lamina. Bar=1 μm.

Figure 3. Photomicrograph of a white area of the carotid artery 6 weeks after balloon catheter injury. The artery is lined by flattened endothelial cells, and the neointima contains numerous layers of smooth muscle cells surrounded by connective tissue stroma. Bar=10 μm.
occasional lipid droplets. In the blue areas, the V, myo of the cells in the inner media and outer media was similar, being 42.7%±3.3% and 46.2%±2.3%, respectively (Table 1). The V, myo, averaged for cells throughout the media, was 44.9%±2.4%, which was significantly lower than that for cells in the control arteries (p<0.01).

**Six Weeks after Balloon Catheter Injury**

At 6 weeks, the extent of endothelial regeneration from the origin of the carotid was similar to that at 2 weeks. Regeneration from the distal end had progressed further, and the isolated white patches observed at 2 weeks had enlarged and coalesced. This shortened the blue-stained region to a variable area that was 2 to 4 cm in total length.

Areas of extensive intimal thickening that contained up to 25 layers of cells were observed. In some areas, the basal portion of the intimal thickening contained numerous layers of cells, while the more luminal areas contained fewer cells and relatively more connective tissue. The connective tissue stroma surrounding the intimal cells appeared to contain greater amounts of elastin than that observed at 2 weeks. The V, myo in the intimal cells was similar in re-endothelialized and smooth muscle-lined areas (55.1%±3.4% and 53.5%±3.0%, respectively, Table 2). These values of the V, myo in the intimal cells in both regions were significantly greater than both values at 2 weeks (p<0.01). Although the intimal smooth muscle cells at 6 weeks had an ultrastructure more like the control cells, they still had a decreased V, myo compared with the control medial cells (p<0.01).

The V, myo in the lining smooth muscle cells of the blue areas was 37.3%±2.5% (Table 2), which was significantly less than that of the cells in the underlying intimal thickening (p<0.01).

In both blue and white regions, the media appeared to contain areas with fewer cells; in some sections, there were not enough smooth muscle cells to provide a full sample of eight micrographs. The V, myo of the remaining cells in both inner and outer media had increased compared to the corresponding values at 2 weeks (p<0.01); the average value for the whole media (62.2%±2.5%) was similar to that of the control media (67.9%±3.6%).

**Eighteen Weeks after Balloon Catheter Injury**

The Evans blue-stained region varied between 2 and 3 cm in total length, with a solid region at the proximal end of the vessels and scattered patches elsewhere. While most of the intimal smooth muscle cells had an ultrastructure similar to the control medial cells (Figure 7), there were still occasional cells with abundant organelles and few myofilaments present, particularly in the Evans blue-stained areas. While the estimates for the V, myo at 18 weeks (Table 2) were numerically higher than those at 6 weeks, there was no significant difference between these values (p>0.05). At 18 weeks, the luminal smooth muscle cells still had a significantly lower V, myo than control medial cells and, in fact, the V, myo was slightly lower than that for the luminal cells at 6 weeks (Table 2).

The media still showed evidence of a re-arrangement of structure. In a few areas, no intact cells were present for analysis. However, the remaining cells throughout the

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**Figure 4.** Transmission electron micrograph of a white area 2 weeks after balloon catheter injury. Most of the smooth muscle cells in the neointima contain abundant synthetic organelles with the myofilaments restricted to small bundles along the cell periphery (arrow). Note the modified cillum in the smooth muscle cell (arrowhead). Bar=1 μm.
Table 2. Volume Densities of Myofilaments in Smooth Muscle Cells in Intimal Thickening and in Luminal Cells of Arteries after Balloon Catheter Injury

| Time after injury | Intimal thickening | Luminal SMC |
|-------------------|-------------------|-------------|
|                   | White             | Blue        | White     | Blue        |
| Two weeks (n=5)   | 38.8±1.0*         | 35.9±3.3*   | 36.3±1.6  |
|                   | (36.0, 41.6)      | (26.6, 45.1)| (32.0, 40.6)|
| Six weeks (n=5)   | 55.1±3.4          | 53.5±3.0    | 37.3±2.5  |
|                   | (45.5, 64.6)      | (45.1, 61.9)| (30.2, 44.3)|
| Eighteen weeks (n=3) | 64.7±2.9     | 58.8±2.0    | 26.7±2.1† |
|                   | (52.3, 77.0)      | (50.2, 67.3)| (17.8, 56.8)|

Each value represents the mean volume density of myofilaments expressed as a percentage of the cell cytoplasm±SE. The numbers in parentheses are the lower and upper limits of the 95% confidence intervals of the means.

n=the number of animals analyzed.

*The 2-week values are significantly different from the 6- and 18-week estimates (p<0.01). †This group was significantly different from the values for the luminal smooth muscle cells at 2 and 6 weeks (p<0.05). There were no significant differences between the volume densities of myofilaments of cells in white and blue areas at any time (p>0.05).

SMC=Smooth muscle cells.

Figure 5. Transmission electron micrograph of an Evans blue-stained region of the injured carotid artery at 2 weeks after injury. The lining smooth muscle cells extend into the underlying intimal connective tissue. Most of the underlying intimal smooth muscle cells contain abundant synthetic organelles and few myofilaments. Bar=1 μm.

Discussion

The results of this study indicate that, after balloon catheter injury, the smooth muscle cells throughout the wall of the carotid artery undergo quantitatively significant changes in ultrastructure. This is summarized in Figure 8. Two weeks after injury, the V<sub>s</sub>, myo in the cells of the neointima and underlying media was approximately one half that of control smooth muscle cells, indicating that most of the cells constituted a phenotypically altered population. Between 2 and 18 weeks, the V<sub>s</sub>, myo of the smooth muscle cells increased toward the level of control medial cells, suggesting that with time there was a shift to a complete reversion. This suggests that the process occurring in the developing intimal thickening and also in the injured media is analogous to the in vitro phenomenon of reversible phenotypic modulation.7,8

Our results are in accordance with previous biochemical studies of the cytoskeleton of intimal smooth muscle...
cells after endothelial denudation. Fifteen days after injury, the intimal cells contained decreased amounts of desmin and increased amounts of vimentin compared with cells of the uninjured media. The cells in the intimal thickening also had a decreased content of actin, with $\beta$-actin becoming the predominant isoform expressed. The changes in the cytoskeleton have also been documented at the mRNA level, with decreased levels of $\alpha$-actin mRNA and of $\alpha$-actin synthesis and a concomitant increase in those parameters for $\beta$- and $\gamma$-actins. These other studies found, as we have, that with time the intimal cells regain the morphological and biochemical characteristics of mature vascular smooth muscle cells.

The formation of an intimal thickening after arterial injury involves migration of smooth muscle cells from the media to the intima. This process is preceded by a sharp increase in the rate of smooth muscle proliferation, initially in the media, and later also in the intima. In vitro studies suggest that, in most instances, a change to a synthetic phenotype occurs before smooth muscle cell division. Furthermore, the expression of a synthetic phenotype by the intimal cells is likely to be important for the production of extracellular matrix components, another important process in formation of the neointima. Although we did not examine smooth muscle turnover in the present study, the observations of cells with an overall highly synthetic phenotype in the intimal thickening at 2 weeks corresponds with previously reported levels of smooth muscle cell turnover, which were approximately 100 times greater than that of the uninjured control artery. It must be noted, however, that only approximately 50% of the cells that migrate into the intima subsequently proliferate. Since the stereological technique used in the present study yields an estimate of the average phenotype of the smooth muscle cells, the results tend to somewhat obscure the observation that cells in the neointima showed a range of ultrastructure. For example, while the majority of cells at 2 weeks appeared to have a highly synthetic phenotype, some other cells that had a higher content of myofilaments were present. This range of phenotypes may be important, since different "subpopulations" of smooth muscle cells may have different proliferative potentials. Thus, at 2 weeks, the cells with a relatively high V, myo may represent the population of cells that migrate into the intima without proliferating or perhaps cells that have undergone only a limited number of replications. The relatively close parallel between the rates of smooth muscle cell proliferation and the changes in phenotype observed here, together with the increased capacity for synthesis of the synthetic-state cells, suggest that these changes in smooth muscle phenotype are important in the formation of an experimental intimal thickening.

With the exception of the cells lining the artery in the Evans blue-stained areas, in time the thymidine-labeling index of the intimal smooth muscle cells declines in all areas of the injured artery. These luminal smooth muscle cells maintain an elevated rate of proliferation for up to 1 year after injury. The present study has shown that these luminal cells remain phenotypically altered up to 18 weeks after injury. It should be noted that, since the

Figure 6. Transmission electron micrograph of the media from an Evans blue-stained area at 2 weeks after injury. The smooth muscle cells contain abundant synthetic organelles, and the myofilaments are restricted to small peripheral bundles. Bar = 1 $\mu$m.
bulk of the cytoplasm of the luminal smooth muscle cells extends into the underlying neointima, a potential problem exists with misclassification of cell profiles just below, but not obviously continuous with, the artery surface. However, since our sampling technique for the intima covered the entire neointima, the contribution of data from misclassified profiles to the overall estimates is minimal. The observation that the V, myo's of the intimal cells did not differ between the white and blue areas at any time after injury supports this suggestion.

Since the phenotype of the intimal cells remained similar in both blue and white areas at all times after injury, this indicates that the modulation and subsequent reversal of phenotype occurred equally in re-endothelialized and in smooth muscle-lined areas. There are two possible explanations for this observation. The first possibility is that both regenerating endothelial cells and luminal smooth muscle cells produce substances that cause reversal of smooth muscle phenotype. In support of this, endothelial cells and contractile-state and postconfluent subcultured smooth muscle cells can produce heparin-like molecules that can inhibit both smooth muscle phenotypic modulation and proliferation. Alternatively, the observed changes in phenotype may occur independently of the lining cells. If the smooth muscle cells are going to respond to the injury stimulus after balloon catheter injury, they become committed to enter the cell cycle within the first 3 days. At later time intervals, the proliferation rate of the intimal smooth muscle cells declines in all

![Figure 7](image)

**Figure 7.** Transmission electron micrograph of the intimal thickening 18 weeks after injury. The intimal smooth muscle cells contain abundant myofilaments and associated contractile apparatus, and the extracellular matrix contains abundant clumps of elastin (arrowheads). Bar=1 μm.

![Figure 8](image)

**Figure 8.** Diagrammatic representation of the changes in smooth muscle ultrastructure after balloon catheter injury in the rabbit carotid artery. The numbers represent the volume density of myofilaments in the cytoplasm of the smooth muscle cells in the control carotid artery and in various layers of re-endothelialized (white) and smooth muscle cell-lined (blue) areas of the injured arteries.
suggesting that the subsequent decline in proliferation rate, and presumably the reversion of phenotype, may occur independent of any inhibitory influences. In conclusion, this study suggests that a reversible modulation of smooth muscle phenotype occurs in the rabbit carotid artery after endothelial denudation. Based on previous in vitro findings,\(^7,8\) we suggest that these changes in smooth muscle phenotype may be an important step in the initiation of smooth muscle proliferation and, hence, neointima formation. A similar sequence of events may also be important in human atherosclerosis. Human DIT near atheromatous plaques contain a population of smooth muscle cells that are phenotypically altered compared to the underlying media (V, myo, 52\% and 72\%, respectively).\(^9\) DIT from arteries free of atherosclerosis, however, show no such difference.\(^8\) A change of phenotype may, therefore, produce a pool of smooth muscle cells in the DIT potentially responsive to mitogens, with an altered ability to synthesize extracellular matrix and an increased propensity to accumulate lipid,\(^27,28\) all of which are important factors in the development of atherosclerotic lesions.

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Index Terms: intimal thickening • smooth muscle phenotype • balloon catheter