Significance of Quiescent Smooth Muscle Migration in the Injured Rat Carotid Artery

Alexander W. Clowes and Stephen M. Schwartz

SUMMARY. Cellular accumulation in the intima of injured artery has generally been attributed to smooth muscle cell proliferation. Since smooth muscle cells in normal artery are found mainly in the media, migration of smooth muscle cells into the intima has been considered a necessary prerequisite for subsequent myointimal thickening. The nondividing medial cells would appear to have no role in the reparative process. We have investigated in the rat ballooned carotid the possibility that nondividing cells might also contribute to injury-induced intimal thickening. All proliferating smooth muscle cells were labeled by 3H-thymidine given by continuous intraperitoneal infusion. The amounts of 3H-thymidine used were not toxic and did not inhibit smooth muscle cell proliferation. Autoradiograms performed on histological cross-sections showed a progressive decrease in the fraction of unlabeled cells at 3, 7, and 14 days after carotid injury. However, the actual number of unlabeled cells remained constant. The calculated growth fraction for the 14-day period was 40%. A substantial number of unlabeled cells was observed in the intima. These data have led us to conclude that only a small fraction of smooth muscle cells in an artery proliferate in response to the injury stimulus, and do so shortly after injury, or not at all. Furthermore, nondividing, as well as proliferating smooth muscle cells, can migrate and contribute, in a substantial way, to the increase in intimal smooth muscle cell number. (Circ Res 56: 139-145, 1985)

THE way in which smooth muscle cells (SMC) accumulate in arterial intima is of considerable importance to our understanding of atherogenesis and arterial wound repair. The generally accepted dogma, at least for injured arteries, is that SMC respond to the injury stimulus by migrating out of the media and proliferating to form a thickened intima. Tritiated thymidine pulse-labeling experiments from our laboratory demonstrate SMC proliferation occurring as early as 2 days after injury in the media (thymidine index ca 30-50%/24 hr) and at 4 days in the intima (thymidine index ca 70%/24 hr) (Clowes et al., 1983a). Similarly, Thomas et al. (1979) have demonstrated in a pig model of atherosclerosis that arterial plaques enlarge on account of cell replication in preexisting intimal cell masses. These data have provided support for the concept that the response to injury and atherosclerosis is principally a proliferative one.

Under circumstances of injury, proliferation and subsequent intimal thickening depend upon early migration of SMC from the media to the intima, as suggested by Hassler (1979) and Webster et al. (1974). SMC migration in response to a variety of stimulants has been studied in vitro (Thorgeirsson et al., 1979; Grotendorst et al., 1981; Inhatowicz et al., 1981; Seppa et al., 1982; Bernstein et al., 1982; Nakao et al., 1982). The results of these investigations demonstrate that SMC proliferation and directed migration are separable phenomena. For example, although SMC proliferate in response to epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin, and platelet-derived growth factor (PDGF), SMC exhibit chemotaxis only in response to PDGF (Grotendorst et al., 1981; Inhatowicz et al., 1981). Furthermore, SMC migration does not appear to depend on proliferation; SMC require both PDGF and other serum factors for proliferation, but require PDGF alone for migration (Bernstein et al., 1982; Weinstein et al., 1981). Since PDGF and other mitogens are probably present at the surface of injured artery in variable amounts, SMC might in some instances be expected to migrate and proliferate, and, in others, to respond by directed migration alone. We have investigated this possibility in injured rat carotid and show that a significant proportion of SMC responding to the injury stimulus can enter the intima without ever proliferating.

Methods

Male Sprague-Dawley rats (500 g; 5 months old) were subjected to left carotid injury by the balloon technique as previously described (Clowes et al., 1983a, 1983b). In brief, under general anesthesia (pentobarbitol), a 2F balloon embolectomy catheter was introduced into the external branch of the left common carotid artery and passed along the common carotid in an inflated state. This procedure effectively strips endothelium off of the luminal surface. During the same anesthesia, an osmotic infusion pump (Alza, Alzet Corp.) containing 3 ml of 3H-thymidine (6.7 Ci/ml, 1 mCi/ml, ICN) was inserted into the peritoneal cavity. This pump infuses continuously at a rate of approximately 5 µl/hr.
At 1 hour and 3, 7, and 14 days, animals were killed and both carotids were retrieved. Two 5-mm segments were cut from the central portion of each carotid for DNA determinations (Clowes et al., 1983a) and a third piece removed, fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline, pH 7.4, and embedded in methacrylate. Cross-sections (2 µm) were cut and autoradiograms prepared (Clowes et al., 1983a) by dipping the slides in Kodak NTB-2 emulsion and storing them in light-tight boxes at 4°C. The autoradiograms were developed after 2 weeks and the thymidine index (%) determined for intimal and medial SMC separately and for entire cross-sections (2 sections/animal). The "unlabeled cell index" (%) for the entire cross section was equal to 100 - thymidine index and the unlabeled SMC DNA at any time point was equal to 1/100 x (unlabeled cell index x total DNA).

The "non-dividing fraction" for each time period (t) was:

\[
\text{Nondividing fraction} = \frac{\text{unlabeled cell index} \times \text{DNA}_t}{\text{DNA}_0}
\]

Where DNA (corrected or normalized DNA content)

\[
\text{DNA}_{\text{left carotid}} - \text{DNA}_{\text{right carotid}}
\]

Growth fraction (%) = 100 - nondividing fraction.

This formulation of the growth fraction corrects for inter-animal variation in normal carotid DNA content and is reasonably accurate as long as the DNA/nondividing cell and nondividing cell volume remain constant. In previous studies, we have shown that the mean DNA/cell remains constant between 0 and 12 weeks after injury (Clowes et al., 1983a). This method of measuring growth fraction also is not influenced by the means used to mark proliferating cells, since 3H-thymidine will not be taken up by nondividing cells. Nevertheless, the possibility that 3H-thymidine infused chronically would alter total SMC accumulation in injured artery was eliminated by including a group of control animals subjected to left carotid injury and receiving continuous pump infusion of lactated Ringer's solution, and experimental animals received 3H-thymidine. The differences between control and experimental carotids are not significant (paired t-test).

A calculation of unlabeled SMC DNA was made for the intima and the media separately at 7 and 14 days after injury. Since the total DNA content of the left carotid could not readily be partitioned into medial and intimal DNA, we made the approximation that DNA_{media} = DNA_{right carotid} and DNA_{intima} = DNA_{left carotid} - DNA_{right carotid}. This approximation was validated by showing that the number of nuclei in the left carotid media per cross-section was the same as in the right carotid media at 3 days (n = 4); left carotid media—288 ± 22 (SD); right carotid media—270 ± 45; at 14 days (n = 5): left carotid media—285 ± 48; right carotid media 265 ± 34. The unlabeled SMC DNA of the media was 1/100 x (unlabeled cell index_{media} x DNA_{right carotid}) and unlabeled SMC DNA of the intima = 1/100 x [unlabeled cell index_{intima} x (DNA_{left carotid} - DNA_{right carotid})].

### Results

Endothelium was completely removed from the left carotid artery by the balloon catheter, leaving only media containing SMC behind. At 7 and 14 days, circumferential intimal thickening was evident. Continuous infusion of 3H-thymidine produced no discernible toxic effect upon the hyperplastic response of the artery to the experimental injury as measured by the increase in DNA content (Table 1; Fig. 1). Satisfactory minipump function was documented at all times out to 14 days by demonstrating labeled cells in fresh skin wounds.

### Table 1

<table>
<thead>
<tr>
<th>Time after injury</th>
<th>1 hr</th>
<th>3 days</th>
<th>7 days</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>3H-Thymidine</td>
<td>1.65 ± 0.37 (4)</td>
<td>5.62 ± 0.79 (5)</td>
<td>8.57 ± 1.57 (5)</td>
<td></td>
</tr>
<tr>
<td>Lactated Ringer's solution</td>
<td>1.40 ± 0.12 (3)</td>
<td>1.95 ± 0.15 (4)</td>
<td>5.15 ± 1.40 (4)</td>
<td>6.63 ± 1.80 (4)</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD (µg DNA/5 mm). Numbers in parentheses = number of animals in each group. The differences between 3H-thymidine and lactated Ringer's groups are not significant (t-test).
made just before sacrifice and by evidence of continuous labeling cells in the epithelial lining of the crypts in gut (data not shown).

Unlabeled cells were observed in both the media and the intima at 7 and 14 days (Fig. 2). The number of unlabeled cells, as estimated from the DNA measurements, in the media declined, and the number of unlabeled intimal cells increased during this period of time (Fig. 3). The possibility that an artefact (asymmetric \(^3\)H-thymidine (\(^3\)H-TDR) distribution in the nucleus, inadequate thymidine labeling) could account for the unlabeled cells was eliminated by preparing autoradiograms on cell dispersions (from collagenase-elastase digested intima alone) and by increasing the concentration of tritiated thymidine in the osmotic pump; in each instance, comparable proportions of unlabeled intimal cells were observed (data not shown). The fraction of unlabeled cells (intima + media) declined from 56% at 3 days to 13% at 14 days (Fig. 4) but the absolute amount of unlabeled cell DNA and the nondividing fraction remained constant (Fig. 5). The calculated growth fraction increased slightly over the time interval (range: 23–39%), but the changes were not significant.

If we multiply the unlabeled fraction by the final left carotid DNA content (normalized for each animal by dividing by right carotid DNA), the result will be the DNA that has not replicated during the 2-week interval of the study. That unlabeled DNA, divided by the amount of DNA at the start of the experiment, provides a measure of the fraction of the cells that never undergo replication. By subtraction from 100%, we obtain an estimate of the growth fraction. Whereas the calculation does make some assumptions regarding DNA content per cell and cell death, these factors should have only slight impact on the estimate of growth fraction. For example, changes in the tetraploid population should be apparent only in the labeled population of SMC—not in the unlabeled cell population, upon which our calculations are based. Since dead, nonreplicating cells would disappear from our estimate of unlabeled DNA, cell death would be expected to produce a falsely low estimate of the nonreplicating population. In summary, our approach produces a minimum estimate of the frequency of nondividing migrating cells.

We estimated the minimum number of cell divisions required to produce the marked increase in DNA content of the left carotid at 14 days. If we use an average growth fraction of 30%, an initial DNA\(_{\text{left carotid}}\) of 1.4 \(\mu g\) (just after injury), a final DNA\(_{\text{left carotid}}\) of 8.57 \(\mu g\) (at 14 days) and we assume no cell death, then each cell would be required to undergo four divisions. Since our previous pulse
label studies indicate that at least the first round of proliferation occurs in the media, no more than three additional divisions could occur in the intima. If the unlabeled intimal DNA mass at 14 days is 0.7 μg (Fig. 3) and the total intimal DNA mass is 6.5 μg (Fig. 1: DNA_left_carotid − DNA_right_carotid), then three divisions (8-fold increase) of an intimal cell mass equal to 0.7 μg would account for the increase. An alternative—but, in view of earlier pulse-labeling data (Clowes et al., 1983a), less likely—possibility is that all the proliferation takes place in the media, and labeled daughter cells migrate into the intima. Thus, we can account for 8/9 of the increase in DNA content of the intima by three rounds of cell replication. The remainder of the DNA must be due to migration without replication. Since the number of these nondividing cells does not increase, we can conclude that, at a minimum, approximately 50% of SMC migrating into the intima subsequently do not proliferate (Fig. 6).

Discussion

In the past, migration from the media through the internal elastic lamina has been considered a necessary prerequisite for introducing SMC into the intima (Hassler, 1970; Webster et al., 1974; Spaet et al., 1975). We now demonstrate that migration alone without proliferation can account for part of the intimal SMC accumulation. Approximately 50% of the cells that migrate into lesions never divide. As the population of intimal nondividing SMC increases, the medial nondividing population decreases. The absolute number of nondividing cells in the vessel wall remains relatively constant in the period between 3 and 14 days after injury, indicating that if SMC are going to respond to the injury stimulus, they do so immediately. Even though the rat carotid artery is thin (three to five medial elastic layers), the SMC growth fraction induced by extensive injury is only between 20 and 40%.
All cells proliferating in response to injury were labeled by \(^{3}\)H-thymidine (\(^{3}\)H-TdR). In vitro continuous labeling, either with \(^{3}\)H-TdR or bromodeoxyuridine, has allowed for measurement of the nondividing fraction over long periods of time (Ross et al., 1978; Matsumara et al., 1979; Rabinovitch, 1983). Although both \(^{3}\)H-TdR and bromodeoxyuridine can produce toxic effects and inhibit cell proliferation, the nondividing fraction should not be altered, since nondividing cells do not take up these molecules. The proportion of the original population of cells in the tissue that never enter the cell cycle can be obtained by dividing the total number of unlabeled cells at the end of the experiment by the number of cells at the beginning of the experiment. Similar studies in vivo have not been possible before, because a method for continuous \(^{3}\)H-TdR administration in small animals was lacking. Since we have quantitative data for DNA accumulation in response to the injury with and without labeled DNA, we can conclude that this dose is not harmful to the normal hyperplastic response. A similar method has been used in vitro by Rabinovitch (1983) to examine the effects of serum and aging upon the human fibroblast growth fractions and transition probabilities out of G0. In vivo, this approach permits a precise measurement of growth fraction, especially as it changes with time and has been used, for example, to quantify proliferation in hepatocytes and oval cells in regenerating liver (Tatematsu et al., 1984).

For the purposes of this study, quiescent SMC in the arterial wall can be considered synchronized in G0–G1. Based on our earlier studies we would expect that the majority of the proliferating cells would enter the growth cycle between 24 and 72 hours after injury (Clowes et al., 1983a). The present data demonstrate that the population of nondividing SMC remains relatively constant between 3 and 14 days after injury, thereby demonstrating that if SMC are going to proliferate, they do so immediately after injury.

The observation that unlabeled cells are present in the intima suggests that SMC can migrate from the media without proliferating. An alternative explanation is that some other nondividing cell type has entered the intima from the blood; however, in injured normolipemic rat arteries (Clowes et al., 1983b; Fishman et al., 1975; Schwartz et al., 1975), only SMC have been observed in the thickened intima. If the unlabeled cells are SMC, then several questions are raised. Do the nondividing intimal SMC have different properties from proliferating SMC which allow for only a limited response (migration only) to mitogens? Are these cells the same as the rest, but because there is a large accumulation of migratory stimulus in the wall compared to mitogen, they migrate without dividing? Are there several different factors introduced into the wall as a consequence of injury—some controlling migration, and, others, proliferation?

At present, there are no data from animal experimentation to discriminate between these possibilities. SMC migrate and proliferate in response to extensive arterial endothelial injury, yet they do not, when the injury is limited (Reidy and Schwartz, 1981). In both circumstances, endothelium is stripped away and the denuded surface covered with platelets. The reason for the difference in response of the medial SMC to these two forms of injury is not known. One possible explanation provided by the studies of Castellot et al. (1982) is that, in the limited-injury model, endothelium adjacent to the narrow denuded strip produce sufficient amounts of heparin-like glycosaminoglycans to inhibit SMC proliferation. Migration might also be affected by these molecules. We have recently demonstrated that SMC migration in vitro is inhibited by heparin (Majack and Clowes, 1984). This effect is dose-dependent, reversible, and specific for SMC, since endothelial cells are not affected.

In cell culture, a variety of mitogens stimulate SMC growth and undirected migration (chemokinesis), but only platelet-derived growth factor (PDGF) induces directed migration (chemotaxis) in a Boyden chamber (Grotendorst et al., 1981). Other factors generated as part of the injury process (platelet factor 4, thromboglobulin, lymphokines, macrophage factors, fibronectin, and collagen fragments) might also play a role (Postlethwaite et al., 1976, 1978, 1981; Gauss-Muller et al., 1980; Wahl and Wahl, 1981; Seppa et al., 1981; Senior et al., 1983). Whole blood serum, but not plasma-derived serum, contains both growth- and migration-stimulating factors for SMC. At least one of these is PDGF. Whether PDGF is important for SMC migration and proliferation in injured artery remains to be determined. Although PDGF induces directed migration in vitro, we do not know whether the concept of chemotaxis is important in vivo in the movement of SMC from the media through the internal elastic lamina to the intima. An alternative possibility is that medial SMC exhibit increased chemokinetic activity in response to the injury stimuli, randomly move about, and occasionally cross into the intima.

We need to consider the possible significance of migration by itself as a mechanism for formation of atherosclerotic lesions. First, the balloon lesion does not appear to be able to provide a model for the monoclonality of human lesions (Benditt and Benditt, 1973). As shown by Pearson et al. (1979), lesions in balloononed hares are polyclonal. This is consistent with the view that randomly migrating medial cells are the source for the cells in the intimal lesion. This leaves open the possibility that occasional migration of individual cells, perhaps occurring during vascular differentiation, could produce a single cell focus in the intima. Such a focus could form intimal cell masses of the sort identified by Thomas et al. (1979) as the site of origin of atherosclerotic lesions in swine or the preatherosclerotic intimal cell masses identified in human coronary
arteries by the Velicans (1979). Second, the reasons for migration without proliferation remain unclear. The Campbells (1981) and, more recently, Gabbiani et al. (1984) have proposed that the loss of contractile phenotype occurs as an early event in lesion formation and is a prerequisite for smooth muscle cell entry into the growth cycle. Perhaps this phenotypic change is necessary for migration but not sufficient for proliferation. The mitogenic stimulus in the balloon model may be present only transiently. Cells that have undergone the requisite changes after this interval may migrate but not proliferate. Perhaps most intriguing is the possibility that some migrating smooth muscle cells, like striated muscle cells (Caplan et al., 1983), may be post-mitotic and lack the ability to divide.

In summary, these experiments provide support for the hypothesis that the injury process stimulates a small proportion of the medial SMC to enter the growth cycle. Although proliferation accounts for much of the intimal accumulation of SMC, migration of both dividing and nondividing cells is also important. This migratory step is probably regulated by promoting and inhibiting factors carried in the blood (e.g., PDGF) and generated locally in the wall (e.g., heparan sulfate).

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