It is now generally accepted that abnormal growth of smooth muscle cells plays a central and characteristic role in the development of atherosclerotic lesions. We need to know the identity of the factors and mechanisms that stimulate this abnormal growth. In contrast, issues surrounding control of endothelial growth are different and less obvious. There is no evidence that excess growth of endothelium contributes to the formation of lesions. There is evidence, however, that endothelial injury leads to proliferation and migration of smooth muscle cells into lesions as a result of the release of platelet-derived growth factor at sites where endothelial continuity is disrupted. Moreover, loss of endothelial integrity is also crucial to thrombosis and important to the lipid insudation that characterizes the development of atherosclerotic plaque. Thus, in the endothelium, rather than being concerned with abnormal growth, we are concerned with growth as a repair process that is necessary to replace endothelial cells lost through normal mechanisms of cell turnover (figure 1). This raises several questions: How is endothelial growth controlled? Does endothelial injury occur in vivo? How does the response to that injury affect the development of atherosclerotic lesions? This review will address those questions in three sections. In the first section, we will review briefly the general principles of cell growth, emphasizing the aspects of growth relevant to the artery wall and endothelium. In the second section, we will deal more specifically with the current state of our knowledge of the control of endothelial growth. In the third section, we will consider the role of endothelial injury and repair in the pathogenesis of atherosclerosis.

General Considerations

Control of Growth by Cell–Cell Interaction

Endothelial cells are different from most other cells in culture. Growth of endothelial cells, unlike that of fibroblasts or smooth muscle cells, is characterized by formation of a highly ordered monolayer. As we discuss below, once cells have formed this structure, the only agents known to be able to stimulate growth are agents that dis-
rupt the continuity of the monolayer. This is true both in vivo and in vitro, and suggests that contact inhibition of growth may be an important mechanism. Contact inhibition of growth, however, remains a controversial and unproven hypothesis in contrast to contact inhibition of movement, which is a well-established phenomenon. Experiments with cultured cells by Abercrombie and more recently by Albrecht-Buehler demonstrated that cell contact is able to inhibit or reverse cell movement. Similar experiments attempting to demonstrate an effect of cell contact on cell division, however, have been difficult to perform. Perhaps the best example is the time lapse study of Martz and Steinberg in which they were unable to find any association between the extent to which a cell was associated with its neighbors and the probability that it would divide. These experiments are not conclusive since the cells were not at their maximum cell density and the period studied may have been too short to allow manifestation of contact-mediated phenomena.

Some data in support of contact inhibition of growth come from studies with cell membranes. Whittenberger and Glaser and Natraj and Datta have presented evidence that cell membranes contain substances capable of inhibiting growth, as would occur during cell contact. To date, however, a specific membrane component with these properties has not been successfully purified. Interestingly, there is also evidence for the appearance of a unique cell surface protein, CSP-60, in quiescent endothelial cells. Again, there is no evidence that CSP-60 functions to control growth.

Another possible role for contact is control of growth by passage of growth-controlling molecules between cells via intracellular channels. This hypothesis is supported by evidence of a loss of communicating junctions in proliferating tissues in vivo. Quiescent endothelial cells also show gap junctions both in vivo and in vitro. Although the frequency of these junctions is diminished in regenerating endothelium, the association may be only coincidental. In summary, while contact inhibition of growth remains an attractive concept, there are no direct experimental data supporting the hypothesis.

**Control of Growth by Nutrients and Growth Factors**

Although there is very little evidence for control of growth of most cultured cells by cell contact, there is considerable evidence that growth can be controlled by factors extrinsic to the cells themselves, including a recently discovered group of hormone-like growth factors. In this view, cell growth stops at a certain density without any direct dependence on the state of cell contact. The limiting components may be nonspecific nutrients, hormones, or specific growth factors. For example, Vogel et al. have shown that the density attained by 3T3 cells is a direct function of the available concentration of platelet-derived growth factor, a specific polypeptide mitogen. In contrast, Holly et al. have proposed that ordinary low molecular weight solutes, such as amino acids, may themselves provide some control of growth. The possibility that such phenomena could exist even in the absence of cell contact is supported by observations of Schubert et al. that anchorage independent cells, grown in suspension, show a similar limitation of growth, depending upon availability of serum.

The relative role of nonspecific nutrients versus growth factors in this process remains unclear, as does the definition of a growth factor itself. It may be worthwhile to point out that growth factors represent a new and still largely undefined component of the body’s endocrine system. These hormones have been derived from a wide and often surprising variety of tissues. Among the defined growth factors, most is known about epidermal growth factor (EGF). EGF may, therefore, represent a model. Originally found in the salivary gland of mice, EGF appears to function as a true hormone in vivo, and is present in serum, urine, and milk. Its activity is modulated in different growth states, as seen in assays both in vivo and in vitro. The factor is a polypeptide with a known sequence and a specific receptor on its target cells. Mutant cells lacking EGF receptors lose the ability to respond to this peptide. EGF has also been shown to regulate the opening of the eye during development and shows an intriguing variation in plasma concentration during pregnancy.

One essential piece of information remains absent. We have obtained neither drug intervention studies nor an example of an animal with a deficiency of epidermal growth factor to allow us to determine whether EGF is essential for normal growth. Such evidence is available for nerve growth factor. In vitro, this polypeptide does not stimulate cell replication and is not, therefore, a mitogen. It does, however, stimulate somatic growth of neurons. In vivo, antibodies to nerve growth factor prevent normal development of the sympathetic nervous system.

Fibroblast growth factor (FGF) is a polypeptide derived from tissue of the central nervous system. As with EGF, FGF stimulates a relatively wide range of cells. Unlike EGF, the distribution of FGF in vivo is unknown, and there is controversy over whether more than one polypeptide has been identified by this name. As we will discuss later, FGF has been extensively studied with endothelial cells.

Another source of polypeptide growth factors is in the medium conditioned by certain cell
Figure 1. Cell cycle. Left: Schematic drawing representing the major events in cell replication. In DNA synthesis, called S, the cells go from a diploid content of DNA (2N) to a tetraploid content (4N). The phase between DNA replication and mitosis is characterized by a 4N content of DNA and is called G2. The interval from M to S is characterized by diploid DNA content (2N) and is called G1. The term G0 refers to this phase of the cycle in a population of actively dividing cells. G0 cells exist in a variety of biochemical states, representing the series of events necessary to go from M to S. Quiescent cells, in most tissues, are also characterized by a diploid DNA content and are thought by many investigators to rest in a characteristic state called G0.

Right: Initiation of growth has an entirely different meaning for the endothelium and for smooth muscle. In the endothelium, growth is necessary to maintain the normal rate of replacement as cells are lost from the surface. Excess proliferation of endothelial cells is known to occur only in cancer and disorders of excess formation of vessels such as retinitis proliferans. In contrast, we have no reason to believe that vascular smooth muscle has any need to replace its normal population. Smooth muscle does, however, show abnormal proliferation and this is thought to be a key event in atherosclerosis.

One factor from cultured cells, MSA (multiplication stimulating activity), has been demonstrated to be related in structure and function to the somatomedins. Other factors, as shown by DeLarco and Todaro and Todaro et al., may be related in structure and function to known growth factors, including the epidermal growth factor.

The most significant growth factor for the artery wall is platelet-derived growth factor (PDGF). Since PDGF is released from platelets, activity is likely to be present in high concentrations at wound sites where one would expect stimulation of growth. Again, PDGF is a polypeptide. There are reports of purified material available in small quantities from a number of laboratories as well as a recent report describing additional mitogenic substances present in the platelet.

Another factor of interest for the vessel wall is derived from endothelial cells. Gajdusek et al. recently presented evidence that endothelial cells make an endothelial-cell-derived growth factor (EDGF), another polypeptide capable of stimulating growth of smooth muscle cells or fibroblasts. This factor or a related component of endothelial-cell-conditioned medium is also angiogenic in the chick chorioallantoic membrane assay. As with FGF, we know nothing of the role or distribution of EDGF in vivo.

Finally, there is also evidence for a growth factor produced by macrophages. This is of interest for the vessel wall because of evidence that macrophages, along with smooth muscle cells, accumulate in atherosclerotic lesions. PDGF, EDGF, and the macrophage-derived growth factor are all able to stimulate growth of smooth muscle cells. Material released by activated macrophages, as well as EDGF, is able to stimulate angiogenesis.

With the exception of the platelet factor, where activity is related to local release, we have little knowledge of how the functions of growth factors are regulated or of what factors determine their availability in vivo. Even in the case of EGF, a highly defined material, there is only limited information on variations in serum concentration with changes in growth state or on the actual distribution of the factor at the tissue level. Mechanisms controlling activity at the cell level are also unclear. There is, for example, no evidence at present that the presence or absence of growth depends upon modulation of the numbers of receptors present. For example, Bhar-
gava et al. have shown that senescent smooth muscle cells, no longer able to divide, have increased numbers of receptors. Similarly, there appear to be constant or increased numbers of receptors in other cell types as they come to a quiescent cell density. While hormonal regulation of growth factor levels remains largely unexplored, there is some evidence for circulating factors with growth effects related to specific organ systems, including the liver and hematopoietic tissues.

Unfortunately, progress in the area of growth inhibitors or chalone has been less rapid than progress in the direction of growth-stimulating factors. At present, there are no purified chalone and no equivalent to the EGF receptor for any specific growth inhibitor. At the level of the vessel wall, Eisenstein and colleagues have derived growth inhibitors for cultured smooth muscle cells. Nam et al. also reported a vessel wall factor that appeared active in inhibiting growth of smooth muscle in vivo. More recently, Castellot et al. found that cultured endothelial cells release a glycosaminoglycan capable of inhibiting smooth muscle growth, but with no effect on endothelial cell replication.

Movement and Replication

Until this point, we have concentrated on general mechanisms of growth control. These mechanisms are most clearly relevant to the growth of cells sparsely plated on a culture dish. For the endothelium of large vessels, however, the growth process of most interest is the regeneration necessary to replace lost cells (figure 1). This phenomenon, wound-associated regeneration, was first studied in culture by Todaro et al. using a razor blade to "wound" monolayers of mouse 3T3 cells. They found that medium able to maintain viable cells, but unable to support proliferation in the confluent, density-inhibited monolayer could support migration and proliferation after wounding. This suggested the possibility that cells at a wound edge either had different growth requirements or were subject to different controls than confluent cells. Later studies with 3T3 cells and other systems, including our own studies with endothelial cells, have shown that some regeneration occurs in serum-free medium. Serum is required, however, for full repair of wounds.

The two components of regeneration, movement and replication, appear to be separable processes. Lipton et al. and Burk found no migration in wounded mouse 3T3 cultures in the absence of serum. They were able to partially purify factors that replaced the required serum. Lipton found two separate serum components supporting wound repair in 3T3 cells. One supported only migration; one supported only proliferation. Burk purified a 45,000 dalton protein from the medium over SV40-transformed baby hamster kidney cells. This protein supported migration of 3T3 cells into a wound and proliferation in the wound as well as in the undisturbed monolayer. Raff and Houck used cytosine arabinoside to inhibit DNA synthesis and found no decrease in migration distance. This showed that it is the active motility of cells that is responsible for the migration, not "pressure" from cells dividing in or near the wound. Similar results were obtained by Gibbins using rat mucosa in organ culture, and by Sholley et al. using X-irradiated endothelial cells in tissue culture.

Cell movement in most cells begins with the protrusion of a broad, flattened portion of the cytoplasm, called the "lamellipodium," away from the main cell body in the direction of cell movement. A common hypothesis for cell movement is that the lamellipodium attaches to the substrate. Translocation of the cell then occurs by retraction of actin filaments associated with the cell membrane. The cell is thus either pulled toward the new edge, or membrane components are translocated along the actin filaments. In support of this, experiments with cytochalasin B show that doses able to inhibit cell motility also disrupt a submembranous web of actin filaments. These doses do not, however, affect the large actin "stress cables" found in the center of the cell, which may play a more structural role.

We have suggested that movement and replication may be causally related, based on studies of the effects of cytochalasin B on endothelial cell regeneration following wounding of stationary density monolayers in vitro. As expected, we found that the ability of cells to move into the wound area was inhibited. When the drug was applied at the time of wounding, however, the cells were not able to progress toward DNA synthesis. If the drug was removed, cells entered S phase with the same kinetics as a freshly wounded culture. These observations suggest that cytochalasin B, either because of its effect on motility or because of some other as yet unknown effect, arrests endothelial cells at a point in the cell cycle close to the critical event associated with stimulation of replication. This interpretation was complicated by a later study of the microtubule-disrupting agents, colchicine and vinblastine. We found that these drugs also inhibited movement of cells into the wound area. There was no evidence of inhibition of replication, however. In fact, there was stimulation of replication not only at the wound edge, but in the intact cell sheet. This surprising result may be explained by the observation that microtubule-disrupting agents also caused a dramatic change in cell shape, including retraction of cell borders followed by a stimulation of replication of cells in...
one of the intact unwounded sheet. In a peculiar sense, microtubule-disrupting agents are "mitogenic" for the endothelium. Thus, it is not clear whether the continued replication at a wound edge in the presence of colchicine is due to failure of the inhibition of movement to be associated with an inhibition of replication, whether it is simply the result of a direct mitogenic effect of colchicine, or whether it is a response to a diffuse disruption of the monolayer.

One particular reason for being interested in endothelial cell migration is evidence that cell movement, but not cell replication, may be strongly dependent upon the medium. For example, Burk et al.81 have found that hyperlipidemic serum selectively inhibits movement of endothelial cells. Our studies confirm similar work by Wall et al.82 on the effects of platelet products. We have found that whole blood serum, when compared to plasma-derived serum, stimulates the movement of endothelial cells (table 1), but has no apparent effect on cell replication either at the wound edge or in cells growing after passage. Thorgeirsson et al.75 were unable to detect stimulation of movement under similar conditions. This may well be due to the sensitivity of the respective assays. Another type of assay system for migration has been employed by Zetter83 to assay the effects of putative tumor angiogenesis factors on capillary endothelial cells. They find that application of tumor-conditioned media enhances the migration of capillary endothelial cells in vitro.

Table 1. Effects of Whole Blood Serum (WBS) vs Plasma-Derived Serum (PDS) on Wound Associated Regeneration

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<tr>
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<th>WBS Non-irradiated</th>
<th>PDS Non-irradiated</th>
<th>2000 R irradiated</th>
<th>PDS 2000 R irradiated</th>
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</thead>
<tbody>
<tr>
<td>Migration distance (mm ± se)†</td>
<td>2.88 ± 0.15</td>
<td>2.1 ± 0.15</td>
<td>2.16 ± 0.2</td>
<td>2.35 ± 0.1</td>
</tr>
<tr>
<td>No. cells in wound per 0.48 mm of wound edge (+ se)†</td>
<td>4 408 ± 46</td>
<td>240 ± 20</td>
<td>199 ± 23</td>
<td>205 ± 18</td>
</tr>
<tr>
<td>Labeling index among all cells in wound (% ± se)‡</td>
<td>4 38.4 ± 3</td>
<td>27 ± 2.8</td>
<td>7 15.6 ± 0.7</td>
<td>18.2 ± 2</td>
</tr>
</tbody>
</table>

*Pairs showing significant differences by t test.
†Six determinations in duplicate cultures.
‡Labeled with 3H-thymidine for 2 hours prior to end of experiment.

Control of Growth at Wound Edges

Hypotheses intended to explain control of replication at wound edges are derived from the more general ideas of growth control described above. One proposed mechanism is the diffusion boundary layer hypothesis of Stoker64 and Stoker and Piggott.65 This posits that cells in the dense region of the culture locally deplete the medium, while sparsely plated cells or cells at a wound edge escape the "diffusion boundary layer" and are exposed to relatively richer medium. This hypothesis is supported by evidence that serum factors are required for wound-associated regeneration. As would be predicted by this hypothesis, Stoker64 found that quiescent monolayers of 3T3 cells are stimulated to grow by stirring or pumping the medium over the cells. He then used cytochalasin B to inhibit migration at wound edges in 3T3 cultures; as the diffusion boundary layer hypothesis predicted, only cells very near the wound edge were stimulated to synthesize DNA.65 This drug does inhibit both movement and replication of endothelial cells, perhaps reflecting a different mechanism of growth control.74 Recent work with 3T3 cells did not support a major prediction of Stoker's hypothesis. According to the hypothesis, thicker boundary layers or higher viscosity medium should inhibit cell proliferation at a lower population density. Neither growth rate nor final cell numbers were lower in media with relative viscosities 5- to 25-fold higher than usual.66

An alternative hypothesis is that wound edge replication results from disruption of cell-to-cell contacts. Some cell types stop growing when they reach certain numbers per unit area and do not grow further even when the growth medium is changed daily. This is most clearly true for the endothelium,87 and seems also to be true for the epithelial lines BSC-1 and CV-1.88 This hypothesis is supported by evidence for a cell surface component able to inhibit growth of 3T3 cells, as already discussed.14-16 It may also be consistent with our observation that colchicine stimulates endothelial cell replication in association with separation of cell contacts.80 Similarly, Baker and Humphries89 observed that the addition of fresh serum causes the release of cell contact and stimulates cell movement and growth in chick embryo fibroblasts. The principal evidence against this hypothesis is Martz and Steinberg's study13 of cells at subconfluent density.

A combination of the effects of cell contact and serum concentration may be needed to explain the results of most wounding experiments. This is supported by the experiments of Burk71 in which he added various amounts of a growth factor isolated from transformed cells to wounded 3T3 cultures. Low doses supported...
growth at the wound edge only; higher doses stimulated cells in all areas of the culture to synthesize DNA. This is similar to other experiments showing that low concentrations of serum are sufficient to stimulate growth of sparse density cultures of 3T3 cells, while higher concentrations of serum are needed to stimulate confluent cultures.

In summary, there is very little known about the actual mechanisms that control movement or replication during regeneration at wound edges. In part, this may result from the diversity of systems studied. For example, data on serum requirements for cells showing a marked dependence on serum concentration for control of cell density may well be misleading when we consider mechanisms for growth control in the endothelium where control of density may be intrinsic to cell-cell interaction and where in vivo concentration of serum is unlikely to vary.

**Protein Synthesis in Regenerating Cells**

Before leaving regeneration, it is important to note that regenerating cells may well have important functional properties that differ from those of quiescent cells. For example, protein synthesis increases in all forms of growth stimulation, including the wound edge response in endothelium. Although there is good evidence that protein synthesis is required for replication, there has been little direct work on the requirement for protein synthesis for cell migration. Gibbins used rat palatal mucosa in organ culture to study the effect of protein synthesis inhibitors on cell migration. There was significant inhibition of both protein synthesis and cell migration. Inhibition of protein synthesis also inhibits migration of endothelial cells at wound edges (figure 2).

The synthesis of one protein, actin, may be of particular interest. Our studies with cytochalasin B suggest that movement, or some other actin mediated event, is critical to the initiation of the cell cycle by regenerating endothelial cells. Benecke et al. and Farmer et al. studied the initiation of the cell cycle in anchorage-dependent mouse embryo 3T6 cells made quiescent by suspending the cells in methylcellulose. Within 3 hours of replating, synthesis of a 43,000 dalton protein, tentatively identified as actin, was resumed at a disproportionately high rate. Exposure to actinomycin D showed that this actin synthesis required new RNA synthesis. Similarly, Riddle et al. and Baker and Humphries observed an increase in cell motility soon after adding serum to chick embryo fibroblast cultures. Folkman and Moscona also described a correlation between cell spreading and cell division using data based on studies of cells on surfaces with differing adhesiveness and data from studies comparing replication of endothelial cells in different parts of a regenerating wound in vitro.

We have very little information about macromolecular synthesis in regenerating endothelium. In vivo, regenerating endothelial cells are characterized by large numbers of both free and bound ribosomes. The identity of the proteins being made within the cisternae, presumably proteins being made for export, remains unknown. One interesting speculation comes from morphologic
studies of regenerated endothelium, as reported by Stemerman and Ross. In their system, the monkey aorta, the normal endothelium at rest lies on a subendothelium containing only very discontinuous fragments of basement membrane. The regenerated endothelium, however, shows much larger amounts of basement membrane material. Wight et al. studied changes in the proteoglycans between normal vessel wall and vessel wall regenerated following balloon catheter de-endothelialization. They found substantial increases in the quantity of proteoglycans and changes in the specific proteoglycans present. Even less is known about the specific identity of the cytoplasmic proteins made by regenerating endothelial cells. Some intriguing data, however, comes from studies of the response of endothelium to hypertension. Gabbiani and his colleagues have used immunofluorescence to examine the content of actin in endothelium during various stages of the development of hypertension in the rat. They found a marked increase in actin content of the endothelial cells during the early stages of development of hypertension. We found that endothelial cells show an increase in replication in this same period. In contrast, Gabbiani and his coworkers found markedly decreased actin content in chronic hypertension. We showed, and Gabbiani confirmed, that endothelial cell replication returns to normal in chronic hypertension. Thus, increased actin content in vivo may somehow be associated with the increased cell turnover associated with hypertension. Actin content has not been studied in other forms of increased endothelial replication.

Endothelial Growth

Regeneration of Endothelium In Vivo vs In Vitro

Unfortunately, there are differences between the sequence of events seen when we study endothelial regeneration in culture and when the same phenomena are studied in vivo. In culture, the sequence of changes is closely parallel to events described for other cell types. Bovine aortic endothelial cells spread processes into the wound area by about 4 hours after wounding. The migrating cells do not show an increase in 3H-thymidine labeling until several hours later. Cells at the leading edge are not always in contact with other cells. Autoradiographic studies show that most of the cell replication occurs in cells that have crossed into the wounded area. In addition, there is some decrease in cell density and increase in replication in areas adjacent to the wound, probably as a result of cell migration (table 2). A significant increase in the frequency of labeled cells can be detected within the intact cell sheet as far as 240 μμ from the wound edge. This may, however, be related to the migration of cells out of the intact sheet and does not necessarily imply that a signal to replicate is being transmitted back from the wound edge.

The sequence of events in vivo, at least in the rat, is somewhat different. First, the time course is different. The peak value for the thymidine index in cultured cells occurred 16 hours after wounding; the peak in vivo was at 48 hours. While this difference may reflect differences in species or undefinable properties of the culture system, differences in the time required to reenter the cell cycle may also reflect differences in the G0 state. Augenlicht and Baserga have reported for WI 38 fibroblasts that the duration of the interval from stimulus to DNA synthesis depends on how long these cells have been maintained in the G0 state. Thus, the longer interval in vivo may reflect a true property of the G0 state in vivo. There are no comparable data for endothelium maintained quiescent for different times in vitro. Second, endothelial regeneration in vivo is polarized. Regeneration in vivo proceeds about six times as rapidly along the vessel axis as it does about the circumference. The reasons for this behavior are not known. The substrate may contain adhesive structures that determine the directionality of movement. Blood flow may determine the ability of cells to move about the axis, or finally, the cytoskeleton of the cells may itself be polarized and may determine the direction of movement. These three ideas are not mutually exclusive, but may be able to be tested in appropriate in vitro systems. Third, and finally, regenerated endothelium in vivo shows a cell density from 150% to 200% of normal. In contrast, regeneration in vitro ends with restoration of normal density. The increase in vivo is a result of a decrease in cell width, suggesting that the hyperplastic property and the polarization of the regenerative response may result from a common factor differing in the in vivo and in vitro systems.
Life Span In Vivo and In Vitro

The basic issue for the endothelium in atherosclerosis is the failure of the proliferation process to maintain the endothelium as a continuous cell layer. One possible explanation of such a failure might be a manifestation of the phenomenon of limited replicative life span. Hayflick first observed that fibroblastic cells derived from human embryo lung lost the ability to divide after approximately 60 doublings. The phenomenon is now well established for all but transformed cells and continuous cell lines. Most studies of endothelial cells in culture show a marked limitation in the number of generations. In our studies, as with those of Levine and Meuller, bovine endothelial cells were capable of replicating approximately 40 times. Duthu and Smith were able to increase this life span by the choice of appropriate media and addition of FGF, but found a limit at less than 90 doublings. In contrast, Buonassisi, using rabbit aorta, and Gospodarowicz et al. using bovine aorta, have reported virtually unlimited life spans. It is unclear whether the latter reports reflect special properties of the culture medium or a spontaneous change in the genome of the cloned cells used by these investigators. Data on replicative life span do not exist at present for endothelial cells in human arteries. We and others have succeeded in getting small numbers of endothelial cells from monkeys or man to grow in primary cell culture (unpublished data). The cells, however, are difficult to passage and rarely will replicate after two to four passages. This may not reflect any real limit; indeed some caution should be expressed about the general concept of finite life span. First of all, the limitation of replicative life span, particularly as demonstrated by human endothelial cells in culture, may be technical rather than biological. Consistent with this hypothesis, Maciag et al. have been able to prolong the life span of human umbilical vein endothelial cells by adding growth factors. Second, the limitations to replicative life span manifest in cell culture may be a result of the random distribution of replicative potential in the culture, rather than a true limitation in the ability of an individual cell to divide. This is consistent with stochastic mathematical models that predict the eventual loss of the ability of cells to divide even in the presence of a portion of the population having unlimited replicative life span.

There are circumstantial reasons for suggesting senescence could occur in the endothelium in vivo. While this is generally a nonreplicating tissue, we have described focal areas with rates of replication as high as 10% of the cells in the rat aortic endothelium being replaced each day. Furthermore, these areas are in regions known to be prone to the development of atherosclerosis. The available evidence in the rat, however, seems to point away from the limited life span hypothesis. For example, when the endothelium is completely denuded from the surface of the aortas of rats, the animals are able to totally regenerate that surface with endothelium arising from the orifices of branches. Since most of the replication can be shown to be occurring at the advancing edge of the regenerating endothelium, this suggests that the cell population is able to undergo a large number of divisions. In contrast, there have been reports of a limited ability

Figure 3. Cessation of cell replication in regenerating rabbit endothelium. Rabbit aortas were denuded by the balloon catheter technique described by Minick et al. (see ref 125). The endothelium was allowed to regenerate for 2 days (A), 2 weeks (B), or 6 weeks (C). At those intervals, animals received three doses of \[^{3}H\]TdR, and tissue was processed for autoradiography (see ref 143). These pictures show endothelium at the cranial ends of patches growing out from intercostal arteries. At 2 days there are numerous labeled endothelial cells and no smooth muscle cells. Arrow indicates the long axis of the vessel. By 2 weeks, only a few endothelial cells (e) are labeled and smooth muscle cells (n) have appeared in the intima. The smooth muscle cells run at right angles to the long axis of the vessel. By 6 weeks only rare labeled endothelial cell nuclei can be seen (lower right), while numerous labeled smooth muscle cells are still apparent. Bar indicates 10 μm.
to regenerate endothelium in the rabbit, a species that also differs from the rat in having a high susceptibility to atherosclerosis. Minick et al.125 showed a limited amount of regeneration as late as 3 months after passage of the catheter. We have repeated those experiments and confirmed the absence of any increase in the area covered by regenerated endothelium between 2 weeks and 6 weeks. Moreover, at 6 weeks, there is no evidence of increased cell replication in the endothelium at the regenerated edge, although the adjacent smooth muscle cells show many replicating cells (figure 3). The mechanism of this arrest remains unknown.

Role of Growth Factors

In this section we will review a rather large literature on factors thought to stimulate endothelial growth. This literature is often contradictory, so we will try to point out phenomena commonly agreed upon. It is also important to express caution about the significance of cell culture data for our understanding of growth of endothelium in vivo. In vivo, endothelial cell growth occurs from an initial state characterized by a high cell density, by the availability of plasma at a concentration of 100%, and by a totally quiescent state with no more than a few cells per thousand replicating each day.121-124 This is true for all known processes of endothelial growth, including new vessel formation seen in response to wounding, inflammation, and neoplasia, as well as the wound edge response seen when endothelium is denuded from the surface of large blood vessels. In contrast, the data presented in table 3 review growth responses over a wide range of cell densities in an often poorly characterized state of quiescence and in the presence of a wide variety of media and serum concentrations. Finally, these studies utilize cells derived from different species, from animals of different ages, and from different vessels.

One example of the problems of interpretation of in vitro data comes from studies of the initiation of growth in quiescent cultures. The maximum density attained by endothelial cells is independent of serum concentration, although the rate of growth of subconfluent cells does depend on serum concentration.66 Once these cells are at saturation density, as they always are in vivo, the addition of more serum or exogenous growth factors does not cause stimulation of growth.66,67 Most of the data represented in table 3, however, represent growth of endothelial cells plated at sparse densities. It is difficult to know the significance of the effects of agents active only at subconfluent or clonal densities when there is no known example of this type of growth in vivo. In contrast, the response of endothelial cells at wound edges has an obvious relevance to in vivo behavior. In our studies,68 we could not detect a requirement for growth factors under these conditions. Indeed, a high percentage of wound edge cells replicate even in serum-free medium.

An important technical issue in growth studies is the origin of the cell type being studied. Most studies have utilized cells from either human umbilical vein endothelium (HUVE) or bovine aortic endothelium (BAE). Some consistent differences between these cells appear in the available literature. Clearly, a central event in the development of culture of both types was the use of proteases to allow selective removal of endothelial cells from the vessel wall.115,120-128 The success of three laboratories in growing HUVE cells may also have reflected the choice of media, particularly the use of concentrations of serum as high as 30%,115,126,128 in contrast, BAE cells show virtually the same extent of growth in fetal calf serum concentrations ranging from 5% to 30%, and some growth can be seen in concentrations as low as 1%. There may also be differences between HUVE and BAE in the importance of cell density. HUVE cells are apparently quiescent when plated in high serum concentrations at low densities of 1.4 x 10^3 cells/cm^2,38,126 whereas BAE cells are capable of proliferation when plated at these densities127,129 (unpublished observations). Finally, there has been no report on cloned HUVE cells, although there have been several reports on clones derived from bovine endothelial cells113,126-131 and from rabbit endothelial cells.118

One factor that is implicit in the last paragraph is that both HUVE and BAE cells have been routinely grown in the absence of exogenous growth factors. This is confusing because of assertions that one factor or another is "required" for endothelial growth. FGF, for example, has been reported by Gospodarowicz and his colleagues129 to be required for clonal growth, growth at low densities, and prolonged replicative life span of BAE. While data from other groups clearly show that endothelial cells can be cloned at low densities without FGF,113 the observation of apparently unlimited life span is limited to those cells propagated in FGF. Thus FGF's effects may relate specifically to cloning and prolongation of life span rather than growth in a more general sense. Related reports indicate that several polypeptide growth factors stimulate growth of HUVE,38,132,133 The control data in these studies show an absence of growth despite the obvious fact that the cells under study were originally obtained and grown in culture without the addition of special growth factors to the basic medium. Again, the effect may result from the plating density used, or the growth factors may have restored the replicative potential lost during earlier passages. In addition, in the case
Table 3. Effects of Growth Factors on Endothelial Proliferation and Regeneration

<table>
<thead>
<tr>
<th>Agent</th>
<th>Human umbilical vein endothelium:</th>
<th>Thymidine labeling index*</th>
<th>Growth†</th>
<th>Wound-associated regeneration‡</th>
<th>Reference</th>
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<td></td>
<td>&lt; 10⁴</td>
<td>+</td>
<td></td>
<td>126, 132</td>
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* Thymidine labeling index: ++ = > 30%, + = 0-30%, 0 = < 1%.
† Growth: + = > 50%, +/− = 30-50%, 0 = < 30%.
‡ Wound-associated regeneration: ++ = > 50%, + = 30-50%, 0 = < 30%.
§ Clonally derived from dermal tissue.
Table 3. (Continued)

<table>
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<th>Agent</th>
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<th>Growth†</th>
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**Abbreviations:** EGF = epidermal growth factor; FGF = fibroblast growth factor; NGF = nerve growth factor; PDGF = platelet-derived growth factor; ECGF = endothelial cell growth factor; EDGF = endothelial cell-derived growth factor; WBS = whole blood serum; PDS = cell-free plasma-derived serum; PMNL = polymorphonuclear leukocytes; sat den = saturation density.

**Symbols:** + = increase compared to controls; 0 = no difference compared to controls; − = decrease compared to controls.

Parentheses indicate contradictory results.

Bold type indicates entries that are discussed in detail in the text.

*Calculated as the number nuclei labeled with $^3$H-thymidine, from 1 to 24 hours continuous labeling.

†Increase in cell number.

‡Starting conditions are confluent cultures with a strip of cells removed.

§Cells cloned and maintained in the presence of fibroblast growth factor.

of FGF, there is a controversy over whether FGF or a contaminating factor is the active agent.38,39

The most interesting growth factors to consider, in the context of this review, are EGF and PDGF. Both of these polypeptides are available to arterial tissue in vivo. EGF is present in serum and therefore available to endothelium at concentrations close to the dose known to stimulate growth of other cell types in vitro.30,33 Gospodarowicz et al.133 have shown that EGF, either alone or in combination with thrombin, stimulates HUVE proliferation. In contrast, unlike HUVE, BAE cell proliferation is not stimulated by EGF and BAE apparently lacks EGF receptors.133 The role of factors derived from platelets has been confusing. PDGF levels in plasma are probably low, but should be increased due to platelet release at sites of endothelial injury.134 Reports from this laboratory,68 as well as by Thorgeirsson and Robertson,10 Davies and Ross,135 and Wall et al.,136 have stated that HUVE and BAE cells proliferate readily in plasma lacking PDGF and other platelet factors. This is true in the wound edge response, as well.68 In contrast, D'Amore and Shepro137 found that BAE cells respond to platelet-derived factors and to whole platelets by higher proliferation rates. Similar reports have appeared for rabbit marginal vein endothelial cells.138 These differences could represent short-lived platelet components other than PDGF or impurities in the PDGF preparation. Most recently, Zetter and Antoniades139 have reported a response to impure, but not to pure, preparations of the platelet-derived growth factor. In conjunction with these studies of growth are the reported enhancing effects of whole platelets on...
the spreading of HUVE. This phenomenon could be related to the suggestion by Folkman and Moscona that endothelial cell migration may be related to stimulation of growth and to our description of inhibition of growth and spreading at a wound edge by cytochalasin B.

One variable that may explain the special growth requirements of sparse endothelial cells is the ability of endothelial cells to condition their own medium. Greenburg et al. have reported that conditioned medium aids survival of endothelial cells plated at low densities. We have found that conditioned medium supplemented with low concentrations of plasma-derived serum supports growth of bovine aortic endothelium in the absence of other exogenous growth factors. The fraction in conditioned medium supporting endothelial cell growth includes material with a molecular weight, based on dialysis, of 1000 to 8000 daltons. This excludes the effects of thymidine, although McAuslan et al. have shown that endothelial cell growth can depend on the concentration of this nucleotide. Furthermore, unlike the endothelial cell-derived growth factor, with a molecular weight of approximately 10,000 to 30,000, the 1000 to 8000 dalton fraction of conditioned medium is not mitogenic for 3T3 cells. Reports by Castellol et al. and Birdwell et al. indicate that conditioned medium from other cell sources are also able to support BAE cell proliferation at either low serum concentrations or at high serum concentrations in the absence of FGF.

Some general statements can be made to summarize these data. Both BAE and HUVE can be grown in culture medium free of detectable platelet-derived growth factor and without the addition of PDGF, FGF, EGF, or other defined mitogens. While plasma is required for growth, this requirement differs from the needs of most other cells in culture, including smooth muscle cells, which have an absolute requirement for one or more defined polypeptide mitogen. The maximum density attained by endothelial cells from both sources is also apparently unaffected by exogenous growth factors or by serum concentration. Once cells have attained this density, growth cannot be stimulated by adding growth factors or serum. Wound-associated regeneration does not appear to depend on any of the known growth factors, although it does require undefined components of serum. Although BAE can be cloned without FGF, it appears that FGF and perhaps other growth factors are important to clonal growth of BAE. The only reports of a replicative life span greater than 100 generations are for cells grown in the presence of FGF, suggesting that FGF may also be important in this form of survival. The significance of clonal growth and replicative life span for the behavior of these cells in vivo are unclear.

Endothelial Injury

Denuding and Nondenuding

At this point we can return to the hypothesis that endothelial injury is the initial event in atherosclerosis. This hypothesis is based on the discovery by Ross and Vogel that a platelet component, likely to be released at sites of endothelial injury and thrombosis, is able to stimulate smooth muscle growth. Surprisingly little is known, however, about the actual loss of endothelium either spontaneously or in response to atherosclerosis risk factors. Studies from our laboratory, as well as those from the laboratories of Wright, Gaynor, Caplan and Schwartz, and Kunz et al. have shown that the basal rate of replication in normal adult animals is extremely low. These studies have also demonstrated areas of the endothelium that show an increased replication. These areas appear to be more frequently associated with branches, although similar areas can be seen at some distance from branches (figure 4).

Endothelial replication is increased in response to hypertension or hyperlipidemia. It is important to point out, however, that areas of increased turnover not necessarily imply areas where the endothelium becomes denuded. With the exception of one report in the pigeon, there have been no convincing reports of areas of spontaneous denudation either in control animals or in animals exposed to hyperlipidemia or hypertension. The only other exceptions come from studies where tissue was examined by the silver stain procedure prior to fixation for electron microscopy or where the endothelial surface was examined well after the initial phases of lesion formation in hyperlipidemia. The problem with this approach is that the silver stain itself may produce changes in the endothelial cell, complicating the evaluation of the extent of injury in the intact animal. Once lesion formation has begun, endothelial changes may represent a result of lesion formation rather than its cause. Recent scanning electron microscopy studies of the endothelium of hyperlipidemic animals have not found evidence of denuded areas in the early stages of hyperlipidemia until lesion formation was advanced.

Lack of convincing evidence for spontaneous denudation does not prove that subendothelium cannot be exposed for brief, repeated episodes. It does, however, make it worthwhile to consider the possible role of nondenuding injuries in atherosclerosis. Nondenuding injuries might contribute to smooth muscle proliferation by alterations in the normal nonthrombogenicity of endothelial cells. Endothelial cells are able to secrete quantities of PGI2 sufficient to inhibit thrombosis. The significance of this property...
for thrombosis on the surface of arteries remains uncertain since blood flow is likely to produce a high dilution ratio. It is also important to point out that nontrombogenicity is not dependent on prostaglandin metabolism alone. Endothelium remains nontrombogenic even when prostaglandin synthesis is inhibited. The only example of "thrombogenic" endothelium is when the cells have somehow undergone neoplastic transformation or are grown under conditions that produce totally abnormal growth patterns. Under these conditions, thrombogenicity seems to correlate with the loss of normal endothelial polarity, an extremely abnormal growth pattern, and the appearance of exoskeletal proteins on the luminal surface. Phenomena of this sort in vivo occur only in neoplasia. Thus, the hypothesis of the thrombogenic endothelial cell remains unproven. For now, we can only relate thrombosis to actual denudation of the vessel surface.

There are at least three other ways in which alterations of a number of endothelial cell functions might lead to smooth muscle proliferation: altered transport, altered platelet interaction, and altered control of growth factor production. Altered bulk phase transport has been shown in regenerating endothelial cells in culture by Davies et al. Similar observations have been made by Vlodavsky and co-workers studying

![Figure 4](image-url)

**Figure 4.** Distribution of replicating endothelial cells on the aortic surface. These figures are maps of the distribution of cells labeling with H^3TdR in the endothelium of the rat thoracic aorta. Each map represents the entire circumference of a segment equal to about one-third of the length of the thoracic aorta. The segments are oriented so that the animals' heads would be toward the left of the page; horizontal borders of each map represent the ventral margin of the vessel. Each dot represents a labeling frequency of 0.1% in a square field equivalent to the area of the reticle used for counting the preparations. The length of one side of this square field, approximately 0.28 mm, is indicated by the scale marks (right). As noted elsewhere, the replicating cells are distributed nonrandomly (see ref 121).
specific transport of low density lipoprotein (LDL) by growing endothelial cells in culture. It is important to note that both of these studies represent uptake, a phenomenon that may not be a good representation of transendothelial transport as it occurs in vivo. If accelerated transport does occur across replicating cells, this could account for localization of lesions at sites of increased cell turnover. Altered transport presumably would not include the platelet-derived growth factor, since that requires platelet release. Other mitogens, however, are available. As shown by Fischer-Dzoga and colleagues, the lipoproteins may contribute to cell proliferation as well as to lipid accumulation. A number of other growth factors, including epidermal growth factor, are normally present in the plasma of man. It is also conceivable that endothelial cells, while maintaining integrity, may be altered in ways that allow altered platelet interaction without adherence. These could include contact with platelets followed by release, uptake, and transport of the platelet factor. In this regard, it is interesting to note that endothelial cells have receptors for other platelet components, including β-thromboglobulin, platelet factor IV, and thrombin. The role of these factors in vivo, indeed, their presence in vivo, remains unproven.

Another possible role for endothelial cells in stimulation of smooth muscle growth is the production of an endothelial cell-derived growth factor and endothelial cell-derived growth inhibitors. The role of these factors in vivo, indeed, their presence in vivo, remains unproven.

Finally, this discussion has emphasized the role of endothelial injury in the proliferative component of lesion formation. The other way in which endothelial injury might contribute to atherosclerosis is via the accumulation of lipid. Relatively little is known about the normal role of the endothelium in this process. It is important to point out that studies of lipid flux have shown that the lipids in the vessel wall are largely derived from serum. Therefore, it is likely that an alteration in transcytosis plays an important role in the pathogenesis of atherosclerosis. We do know that endothelial cells, like other cells, possess high affinity receptors for LDL. It is also known that LDL is capable of endocytosis in culture and show increased endocytosis when growing or regenerating. We do not know, however, whether the endocytotic process observed in cell culture is the same as the mechanism involved in transport of LDL across the endothelial cell into the vessel wall. Alternatively, lipid accumulation may be affected by the composition of the connective tissue beneath the endothelium. Wight has shown that there is an accumulation of proteoglycans in atherosclerotic vessels at sites of endothelial regeneration. These proteoglycans are known to have a high affinity for LDL and thus may cause accumulation by increasing the affinity of the vessel wall for lipoproteins rather than increasing the entry rate.

**Critical Denudation Period**

If we assume that injury does occur via denudation, it is possible to make some statements about the probable duration of exposure of the thrombogenic subendothelium and about the interval of denudation required for stimulation of smooth muscle proliferation.

Studies in our laboratory and elsewhere have attempted to define the time course of the regeneration process following removal of the endothelium. Our studies in vivo and in vitro have examined the response of rat aorta to denudation. As in other animals, denudation by the balloon technique leads to a characteristic intimal accumulation of smooth muscle cells. At least in the rat, smooth muscle lesions are formed only in those areas requiring more than 7 days for endothelial regeneration. This suggests that there is a "critical lesion size," i.e., a critical amount of endothelium that must be removed before smooth muscle lesion formation will be stimulated. Work by Hirsch and Robertson and Reidy and Schwartz confirmed this by showing that small areas of endothelial denudation two to 20 cells wide do not result in smooth muscle proliferation. Thus, it appears that brief exposures to platelet releasate are not sufficient to stimulate smooth muscle proliferation. Moreover, recent reports by Groves et al. and Goldberg et al. based on the use of the balloon catheter in rabbits indicate that most of the platelet adhesion occurs in the first 24 hours following experimental denudation. Release of granules, which contain platelet-derived growth factor, into the vessel wall can only be detected in the first 4 hours after denudation. It is interesting to speculate whether the nature of the platelet interactions in the days after initial exposure may play a special role in the stimulation of smooth muscle proliferation or whether an accumulation of platelet-released materials may be required over a longer time period.

Reidy, in this laboratory, attempted to make a more reasonable model for spontaneous cell loss by removing strips of endothelium only one to two cells wide. These wounds completely closed within 6 to 8 hours, without stimulation of DNA synthesis in the endothelium. The rapidity of this response suggests the possibility that regeneration might be coordinated with endothelial desquamation. This would explain the paradox of cell replication studies that show focal areas of high cell turnover, while scanning electron microscopy studies continue to show a continuous endothelium. Recent work from our labo-
VASCULAR WALL GROWTH CONTROL

Summary

The current state of our knowledge of the control of endothelial growth and the role of endothelial injury in the pathogenesis of atherosclerosis can be summarized as follows:

1. Endothelial cells can be grown in plasma-derived serum in the absence of exogenous growth factors. This is quite different from the growth requirements of most other nontransformed cells. These factors may, however, prolong replicative life span and increase the ability of endothelium to grow at sparse density. The relevance of these phenomena to the control of endothelial growth in vivo is unclear. There is no evidence that exogenous growth factors are required for wound edge regeneration. In view of the relative lack of growth factor requirements, it is intriguing to consider the possibility that the critical control factor for endothelial cell growth is cell contact.

2. Endothelial cell regeneration may be dependent on endothelial cell motility. The nature of this relationship may be important in controlling the ability of the endothelium to regenerate itself under different flow conditions around lesions or in different parts of the vessel tree and in determining the ability of the endothelium to respond to changes in the connective tissue overlying lesions.

3. Endothelial cells in vivo are able to regenerate small areas of denudation extremely rapidly. This process may be sufficiently rapid to permit the endothelium to replace dying cells as they are being lost, resulting in desquamation without denudation.

4. We have little evidence for endothelial denudation either spontaneously or in response to atherosclerosis risk factors until after lesion formation has begun. This does not rule out the possibility that small, repeated, transient episodes of denudation occur and play a role in the initiation of atherosclerotic lesions. It is important, however, to begin considering the role of nonnondending injuries in atherosclerosis.

5. The fact that thrombosis occurs in atherosclerosis implies an eventual breakdown of endothelial integrity. The mechanism of that breakdown remains unknown.

6. Finally, there is the question of interactions between smooth muscle cells and endothelial cells at the level of growth control. This includes the evidence that there is a critical amount of endothelium that must be lost before lesion formation is stimulated and the recent evidence that endothelial cells produce substances able to regulate growth of smooth muscle cells.
Acknowledgment

We thank Russell Ross for his wise and constructive criticism.

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