

# THE SMOOTH MUSCLE CELL

## II. Growth of Smooth Muscle in Culture and Formation of Elastic Fibers

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### ABSTRACT

Smooth muscle derived from the inner media and intima of immature guinea pig aorta were grown for up to 8 wk in cell culture. The cells maintained the morphology of smooth muscle at all phases of their growth in culture. After growing to confluency, they grew in multiple overlapping layers. By 4 wk in culture, microfibrils (110 Å) appeared within the spaces between the layers of cells. Basement membrane-like material also appeared adjacent to the cells. Analysis of the microfibrils showed that they have an amino acid composition similar to that of the microfibrillar protein of the intact elastic fiber. These investigations coupled with the radioautographic observations of the ability of aortic smooth muscle to synthesize and secrete extracellular proteins demonstrate that this cell is a connective tissue synthetic cell.

### INTRODUCTION

With refinement in tissue culture methodology, it has been possible to grow many different types of cells and to retain their state of differentiation.

Several attempts have been made to grow smooth muscle cells in culture. Tissues used as a source of smooth muscle have included aorta and uterine myometrium, both of which have produced an outgrowth of cells that have the phenotypic appearance of smooth muscle (1-3).

This report presents observations of the growth in cell culture of smooth muscle derived from the media and intima of the guinea pig aorta, and demonstrates that the smooth muscle cells retain their differentiated state at all times during the culture period. The formation of elastic fibers by subcultures of these cells is presented.

### MATERIALS AND METHODS

Segments of both abdominal and thoracic aortas were obtained from prepubertal guinea pigs by

careful dissection under ether anesthesia. The segments of aorta were placed in a depression slide containing tissue culture medium, after which the adventitia and the outer portion of the media of each segment was carefully removed under a dissecting microscope. Segments of the aorta which were representative of those used for culture were fixed in a mixture of 2% paraformaldehyde-2.5% glutaraldehyde in cacodylate buffer (pH 7.3) (4) and prepared for both light and electron microscopy. The remaining inner portion of the media and the intima were removed to a separate dissecting dish and washed several times with fresh culture medium. At this point each segment was cut into approximately 1-mm squares and placed on glass slides to be used in Leighton tissue culture tubes. Each slide was inverted so that the segments of tissue were located at the interface between the slide and the bottom of the glass tube. 2 ml of culture media was carefully added to the tube so that no air bubbles formed and so that dislodgment of the tissue was avoided.

The tubes were capped loosely and placed in a moist tissue culture incubator at 37°C containing an atmosphere of 95% air and 5% CO<sub>2</sub>. After several hours, to insure equilibration of the atmosphere within each tube, the tube caps were tightened and the tubes were placed in a dry, air-flow incubator at 37°C. They were not disturbed for the first week, at the end of which they were fed with fresh medium.

The cells which grew from the explants had become relatively confluent within a period of approximately 4 wk. They were then rinsed with a Versene buffer solution, and subsequently trypsinized with a solution of trypsin in Versene buffer (Difco Bactotrypsin 1:250-0.05% solution, Difco Laboratories Inc., Detroit, Mich.) by incubating them in this solution for 5 min at 37°C. The resulting suspension of cells was pipetted into a 6 oz culture bottle containing 10 ml of the Dulbecco-Vogt modification of Eagle's medium (5) and incubated as mentioned previously. The medium was changed the next day to remove trypsin and cell debris.

6-cm plastic Falcon Petri dishes (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) were used for growing large numbers of cultures for extraction purposes as well as for examination by electron microscopy.

Bottles containing cells which had previously become confluent were used as the source of the subcultures employed in this study. To accomplish this, the bottles were trypsinized as mentioned previously and the resulting cell suspension was placed in a centrifuge tube containing twice the original volume of medium present in the bottles. The cells were spun down gently and the supernatant was decanted. The cells were then resuspended in fresh medium, and a sample was counted in a counting chamber. These cells were then added to 60 mm Falcon tissue culture dishes and the volume of medium was brought to 4 ml. Approximately 50,000 cells per ml were placed in each culture dish. These were subsequently fed twice weekly.

All of the growth experiments were performed in dishes containing cells derived from the ninth subculture or later.

The dishes of cells were fixed for examination by electron microscopy at weekly intervals for the first month, and after 6 wk, 8 wk, and 10 wk in culture.

### *Tissue Culture Media*

The Dulbecco-Vogt modification of Eagle's (5) medium was modified in that it contained 3 ml of 7.5% sodium bicarbonate, 1.0 ml of nonessential amino acids and 1.0 ml of sodium pyruvate, and 10% newborn calf serum and 0.5 ml penicillin.

### *Preparation for Electron Microscopy*

Cells for examination by electron microscopy were grown either in the Falcon plastic culture dishes as described previously, or in identical dishes in which a carbon coat had been placed on the bottom of the dish in a vacuum evaporator. These carbon-coated dishes were subsequently sterilized by placing them close to a UV light source for 20 min. At the time of fixation, a volume of fixative, equal to the amount of the media in the culture dish, containing 4% paraformaldehyde and 5% glutaraldehyde in cacodylate buffer (pH 7.3) (4) was added to the cells *in situ*. They were fixed at room temperature for 20 min. The cells were then washed with fresh culture medium for 15 min and postfixed for 30 min at room temperature with a 2% osmium tetroxide solution buffered with *s*-collidine at pH 7.3. They were dehydrated in a graded series of ethanol for 15 min and infiltrated with Epon 812 directly in the Petri dish. No propylene oxide was used in this procedure. The Epon was allowed to partially polymerize in each culture dish at room temperature overnight until it became somewhat viscous. At this time Beem capsules (Ted Pella, Altadena, Calif.) were completely filled with similar viscous epoxy resin and inverted over the cells in the dish so that the series of inverted capsules containing resin filled the culture dish. The dish containing the inverted-filled capsules were polymerized in the 60°C oven for 48 hr. At this time each capsule was removed from the culture dish by grasping it with a pair of pliers, twisting it, and pulling it straight out while keeping it perpendicular to the surface of the dish. This procedure produced a flat surface at the end of each capsule containing a carbon film under which lay the cells which had grown in the dish. It is important to note that when the cells were grown in a dish not containing a carbon film, it is necessary to remove the capsules from the dish immediately after it is removed from the oven. Removal of the capsules will be more difficult if the dish is allowed to cool.

The cells could be easily viewed in a dissecting microscope and the blocks could be trimmed to the areas which were to be sectioned.

### *Elastic Fiber Extractions*

20-30 dishes of cells were harvested by scraping the dishes with a rubber policeman. This tissue was homogenized in 5 M-guanidine hydrochloride, and an elastic fiber extract was prepared by using the method previously described by Ross and Bornstein (7).

## OBSERVATIONS

### *Characteristics of Cells in Culture*

The cells grew out slowly from the original explant and had a doubling time of approximately

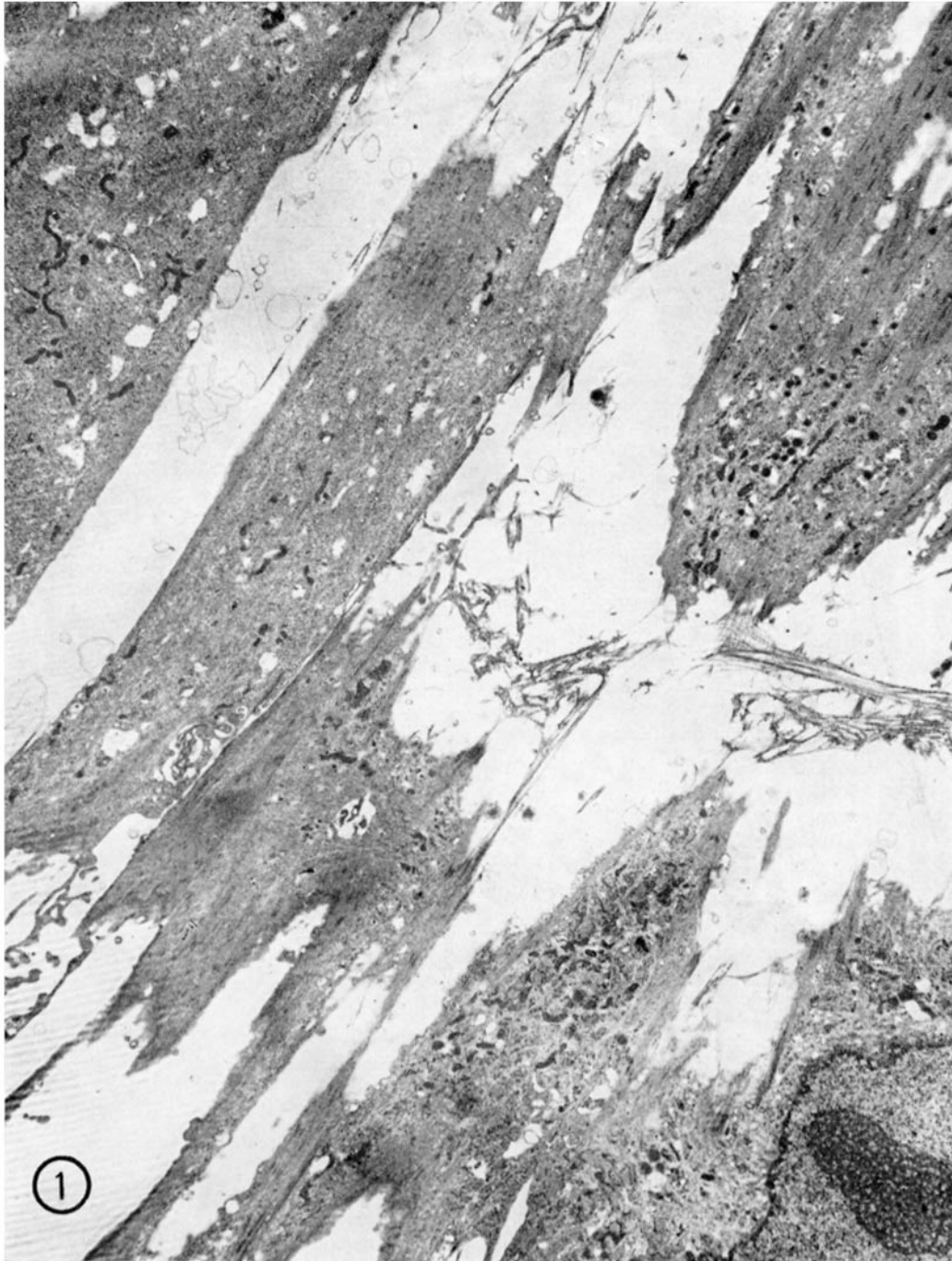


FIGURE 1 A low power electron micrograph of a tangential section of part of a smooth muscle culture that is 8 wk old. The cells can be seen to be rich in myofilaments and dense bodies together with numerous mitochondria and other organelles. The cell in the lower right corner contains a prominent nucleolus within its nucleus. Cell processes bring the cells in contact with one another.  $\times 3700$ .

48 hr. This was apparent after subculturing in 60-mm plastic Falcon Petri dishes, in that it took approximately 1 wk for 50,000 cells per ml to become confluent and begin to pile upon one another in the dish. The cells did not appear to grow in a distinctive pattern in culture, except that it was clear that they grew in multiple overlapping layers, and did not form swirls as is characteristic of fibroblasts derived from the same source. The shape of the cells was varied. In general, they were quite large and contained a

centrally-placed nucleus with several prominent nucleoli. In phase optics, the cells could be seen to have large areas of cytoplasm which contained a series of parallel lines presumably relating to bundles of myofilaments. The cells would sometimes have long, narrow cytoplasmic processes which ran from one cell to the next (Fig. 1).

#### *Fine Structure*

At all of the times examined during growth in culture, the cells retained the phenotype of differ-

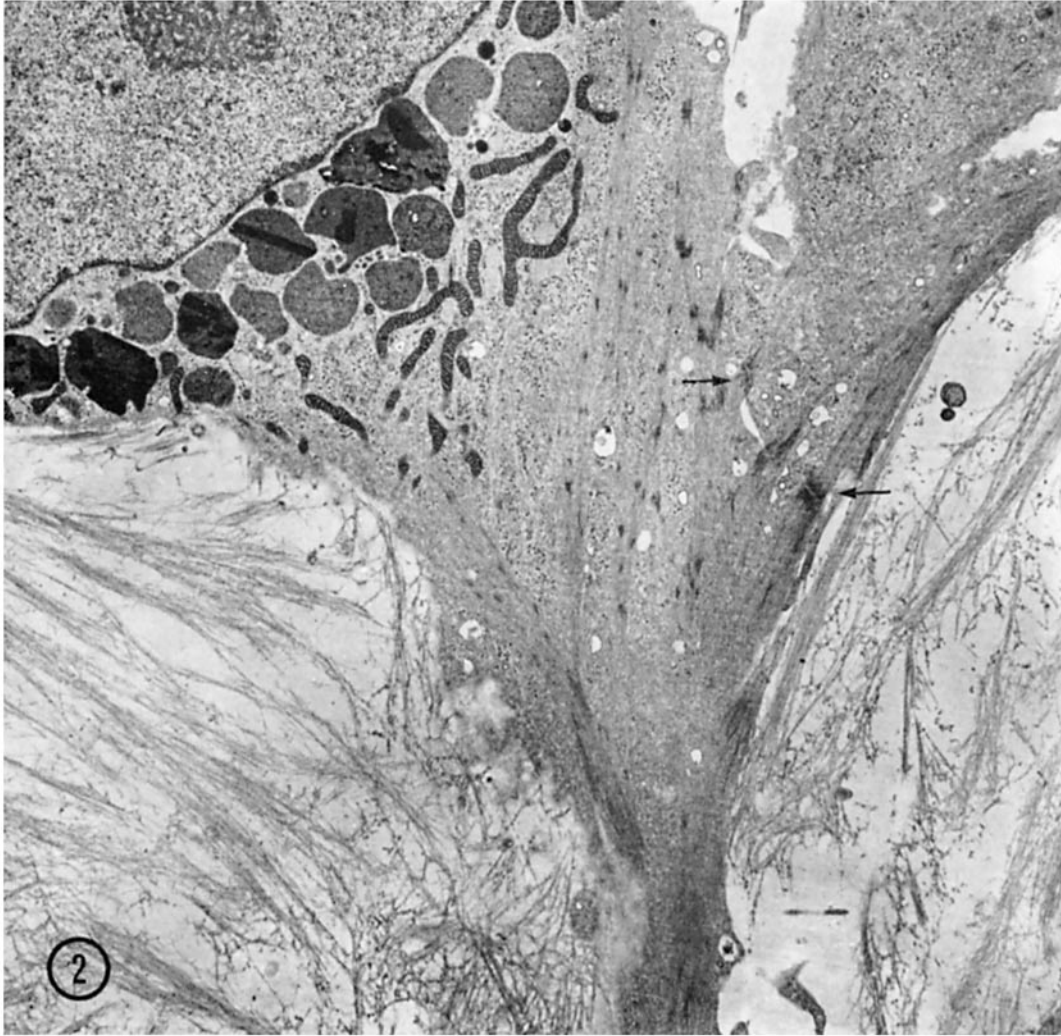
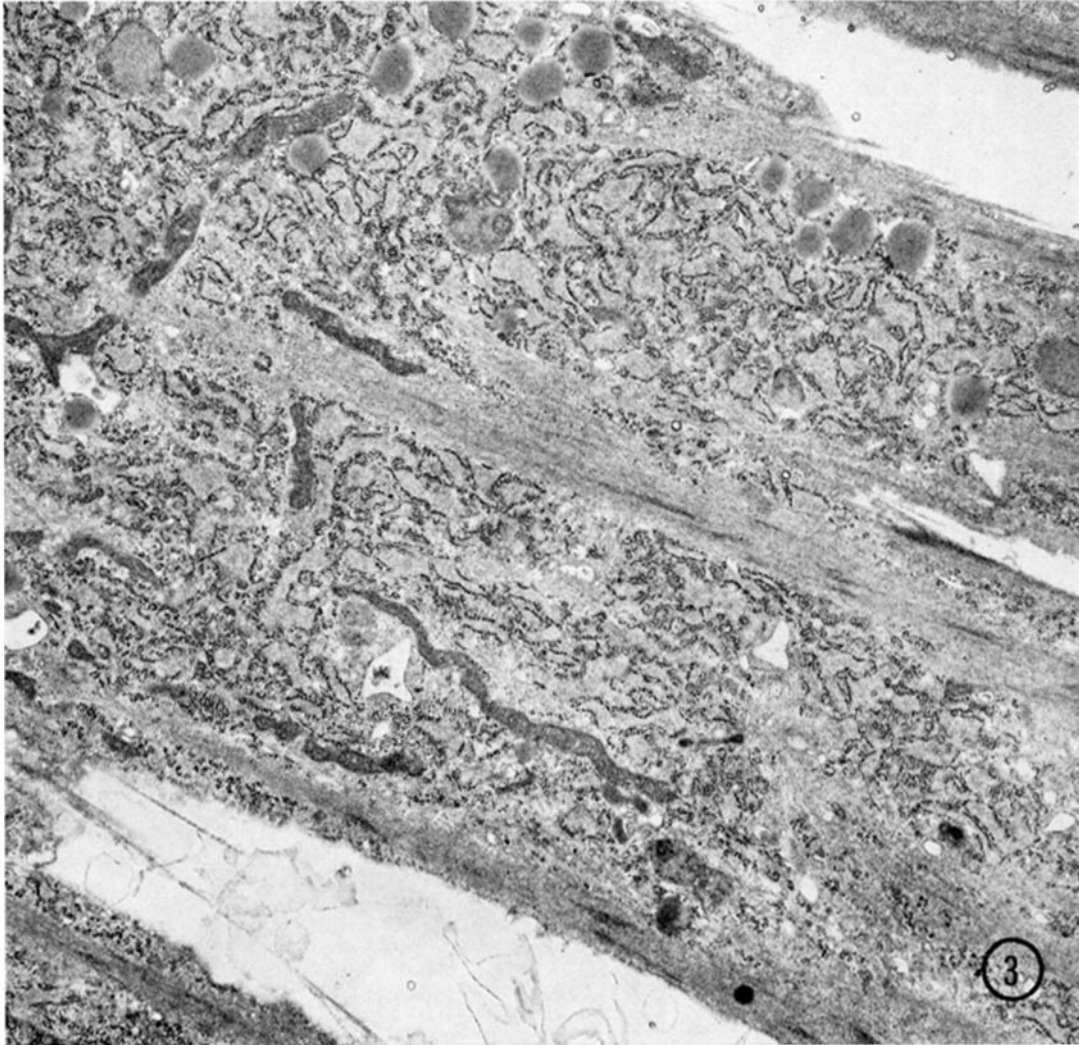


FIGURE 2 This micrograph of an 8-wk-old smooth muscle culture shows parts of two cells which are attached to each other in several regions (arrows). These attachment sites are reminiscent of the kinds of attachments seen between muscle cells. The extracellular spaces contain many microfibrils. The cell contains numerous dense bodies and a branched mitochondrion. The dense bodies may represent autophagic vacuoles.  $\times 6000$ .

entiated smooth muscle. Each cell contained large bundles of filaments resembling myofilaments, approximately 60 A in diameter, which coursed throughout their cytoplasm. Large numbers of dense bodies, or "attachment plaques," were found throughout the bundles of myofilaments. The myofilaments filled the cell wherever there were no organelles (Figs. 1-4). The cells often contained a well-developed rough endoplasmic reticulum and Golgi complex together with numerous large, sometimes branched mitochondria (Figs. 2, 3). After longer periods of growth in

culture, they contained many membrane-bounded, dense bodies (Fig. 2). When sectioned appropriately, numerous vesicles could be seen at the periphery of the cells, close to the cell surface (Fig. 4).

The ultrastructural characteristics of the cells were the same at every point in time during their growth in culture. The relative volume occupied by organelles varied from cell to cell, in that some cells contained more rough endoplasmic reticulum than others; however, all of the cells were extremely rich in cytoplasmic filaments.



**FIGURE 3** A portion of a smooth muscle cell in culture demonstrates that this particular cell has an abundant and well-developed rough endoplasmic reticulum. Wherever there are no organelles such as the reticulum, mitochondria, or lipid deposits, the cytoplasm is filled with filaments containing dense bodies.  $\times 10,000$ .

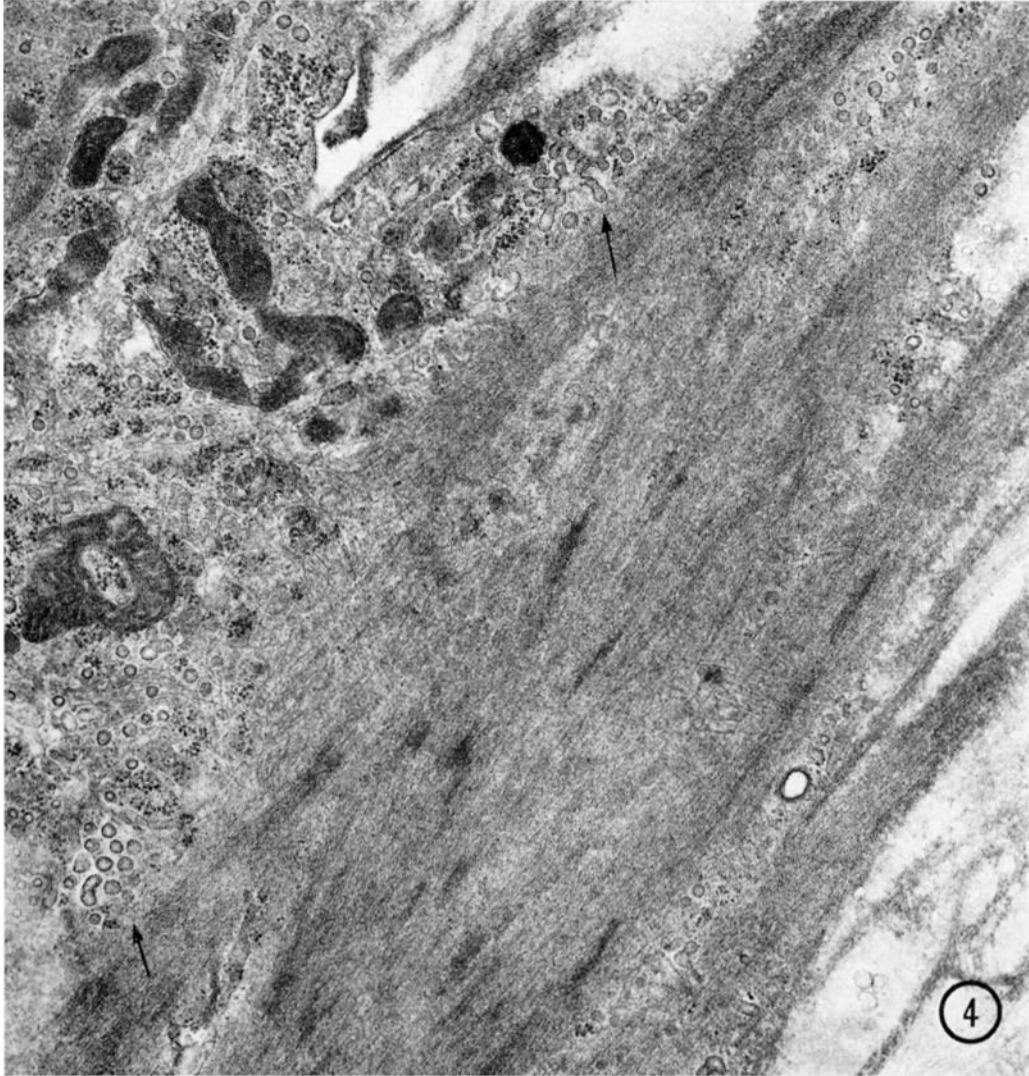


FIGURE 4 A higher magnification of a portion of one of the smooth muscle cells from a 6-wk-old culture demonstrating the large numbers of filaments and dense bodies within its cytoplasm. At the periphery of the cell, numerous vesicles, some of which appear to be either merging with a common source or developing from it, can be seen (arrows). Mitochondria and some rough endoplasmic reticulum are visible as well.  $\times 20,000$ .

Attachment sites were commonly seen between the smooth muscle cells in culture. These took the form of intermediate type junctions which often demonstrated regions of increased density immediately adjacent to the plasma membranes of the cells (Fig. 2). In cross sections, other sites of attachment could be seen which were similar in appearance to gap junctions (8) (Fig. 6).

Examination of cross sections of the cultures showed that the cytoplasmic filaments were uni-

form in diameter (Fig. 8) and were typical in appearance to those of smooth muscle sectioned transversely.

#### *Elastic Fiber and Basement Membrane Formation*

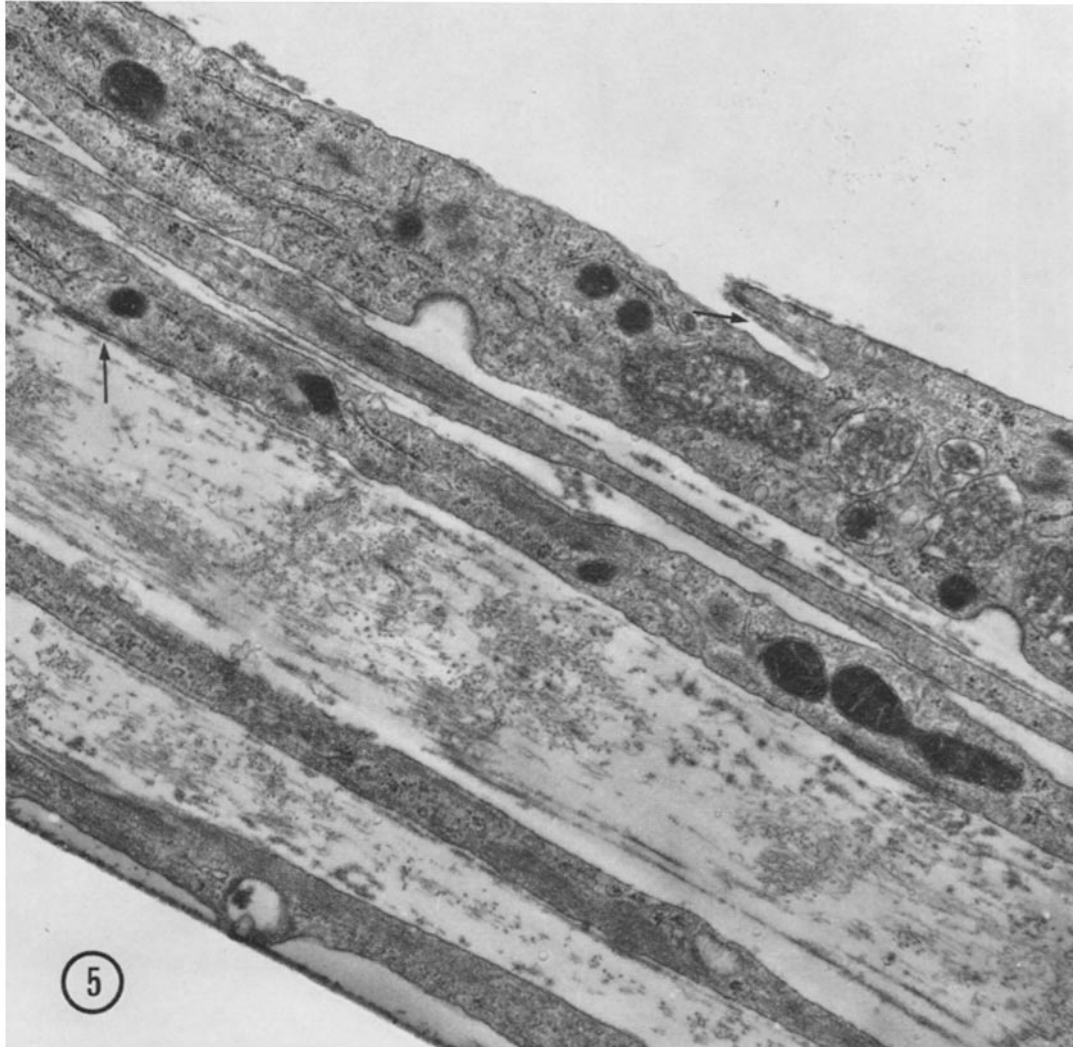
During the first 1-2 wk of growth in culture, extracellular material, reminiscent of basement membrane, began to form in the spaces between

the cells and along the cell periphery. This substance appeared as a feltlike meshwork of amorphous material containing a few very fine filaments (Figs. 5, 7, and 9). At approximately 3 wk of growth in culture, 110 Å microfibrils appeared within the extracellular spaces both immediately adjacent to the cells and at varying distances from them. These microfibrils were identical in appearance to elastic fiber microfibrils previously described and characterized by Greenlee et al.

(6) and Ross and Bornstein (7) (Figs. 5, 7, and 9).

Cross sections of the cultures showed that the microfibrils and the basement membrane-like material were present at several layers between the cells (Figs. 5 and 7).

The numbers of microfibrils and amount of basement membrane-like material increased during the longer periods of growth in culture, and after 7–8 wk they filled relatively large segments



**FIGURE 5** This micrograph demonstrates a cross-sectional view of a 6-wk culture of smooth muscle cells. The bottommost cell rests on a carbon film. Between three of the layers of cells, microfibrils similar in appearance to elastic fiber microfibrils can be seen. Material reminiscent of basement membrane (arrows) is also visible around many of the cells. The microfibrils can be seen at higher magnification in Fig. 7.  $\times 20,000$ .



FIGURE 6 Two smooth muscle cells from a 6-wk-old culture demonstrating a site of attachment between the two cells similar in appearance to a gap junction. The myofilaments in the bottommost cell and the well-developed rough endoplasmic reticulum in the upper cell are characteristic of the cells in the culture.  $\times 60,000$ .

of the extracellular space. At this time small globules of material, which were amorphous in appearance, and which had no affinity for either lead or uranyl acetate, began to appear within the extracellular spaces. They were often found in close apposition to both the surface of the smooth muscle cells and to the extracellular microfibrils (Fig. 10). These globules were identical in appear-

ance to the globules of elastin which have been demonstrated to form in vivo in elastic fibers (6).

#### *The Microfibrillar Protein*

After 6 wk of growth in culture, large numbers of cells were harvested for extraction of elastic fibers. The cells were scraped from a series of



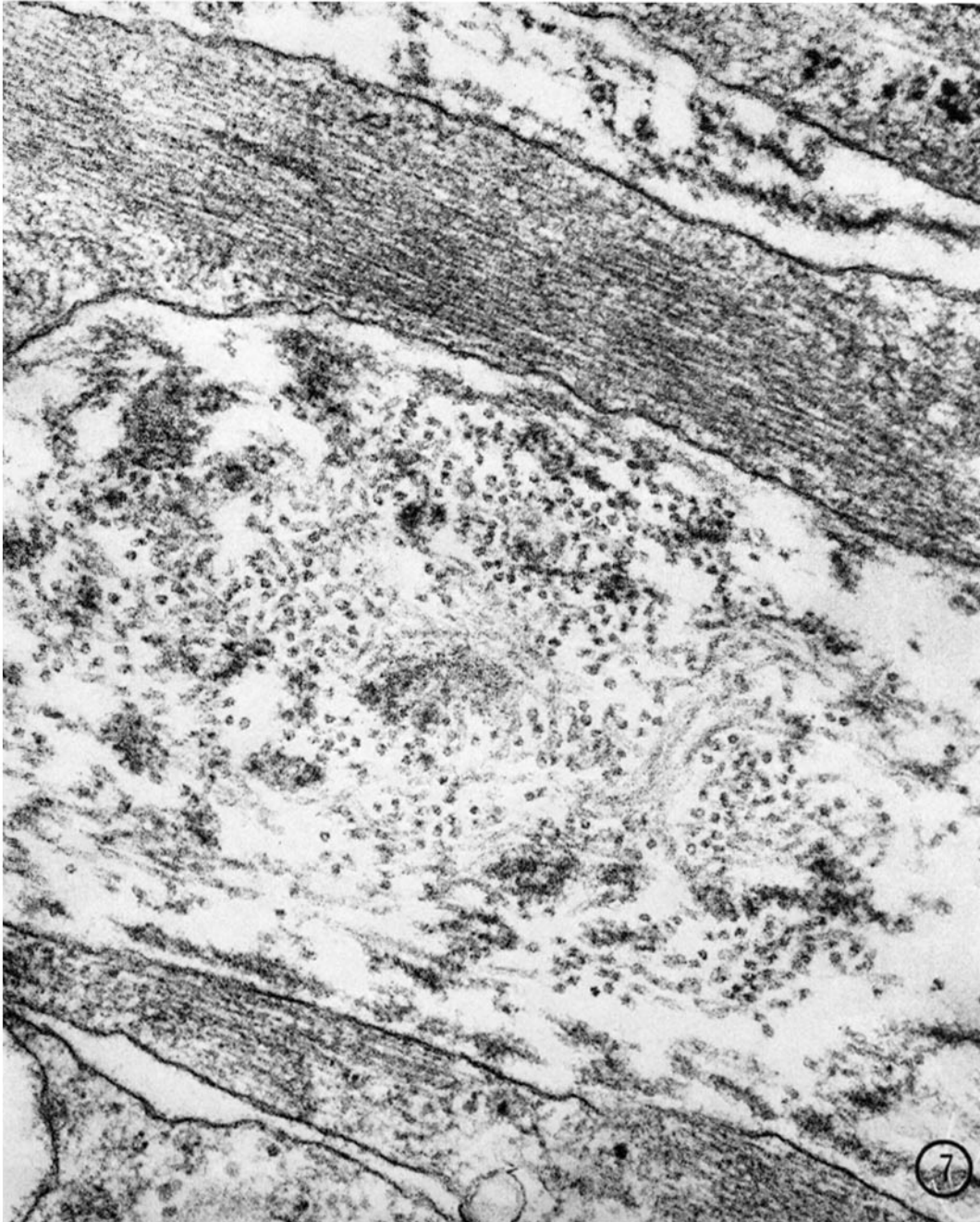


FIGURE 7 In this micrograph, several of the smooth muscle cells from a culture have been sectioned transversely. The elastic fiber microfibrils seen between these cells are characteristic of elastin fiber microfibrils in vivo. They could be found at numerous levels between the cells in culture.  $\times 88,000$ .

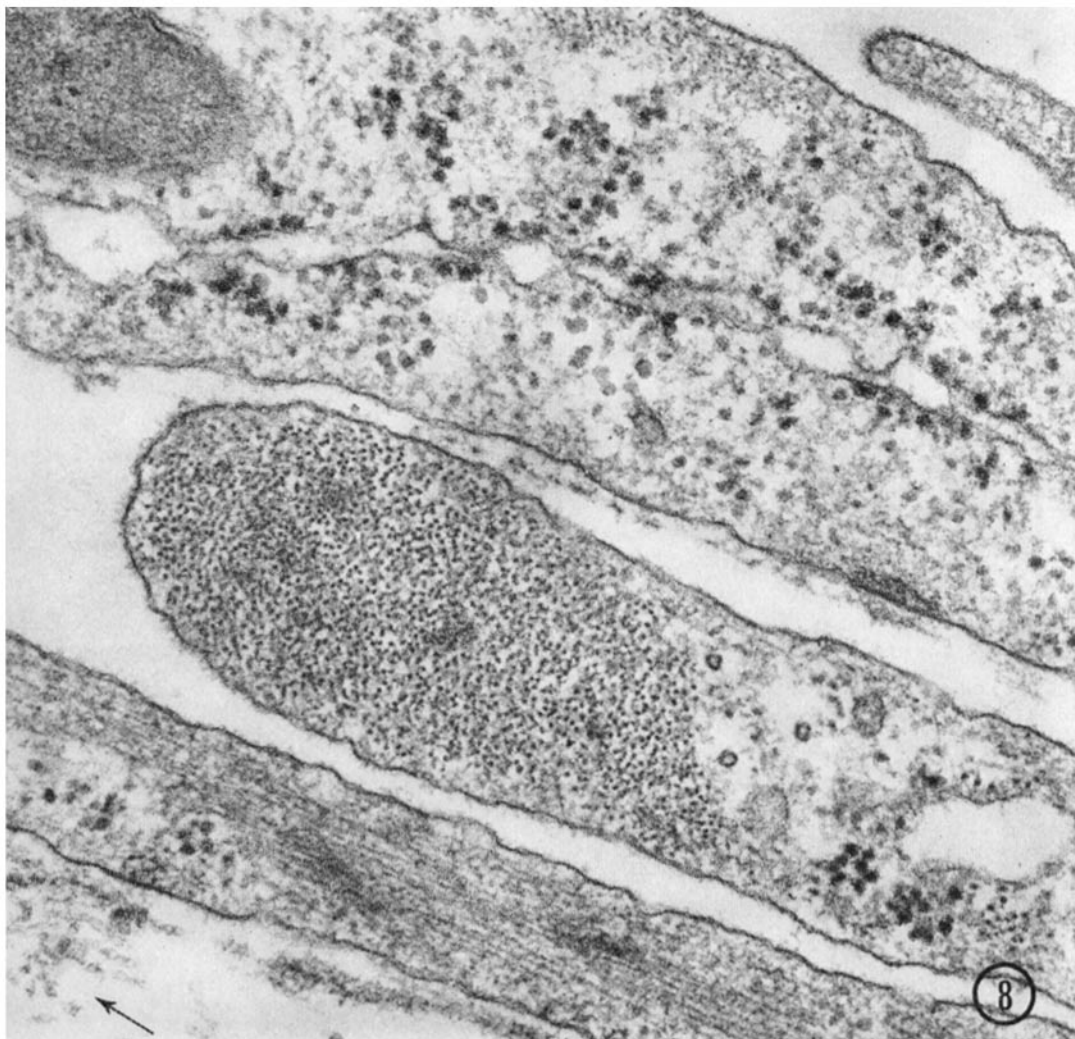


FIGURE 8 In this transverse section of a 6-wk-old culture, the myofilaments can be seen within the cell in the center of the micrograph. They are approximately 60 Å in diameter and are sectioned longitudinally in the lowermost cell. Wisps of material similar to basement membrane can be seen adjacent to two of the cells in the micrograph, and a few extracellular 110 Å microfibrils (arrow) are present in one corner.  $\times 80,000$ .

24–36 Petri dishes and the elastic fibers and cells were homogenized and extracted in guanidine hydrochloride, followed by collagenase (7). Electron micrographs of the samples of the pellet obtained after extraction, which were subsequently used for amino acid analysis, demonstrate that this pellet was rich in microfibrils, identical in configuration and staining characteristics with those obtained from intact elastic fibers (Fig. 11). Amino acid analyses of these extracts showed that the microfibrillar protein was similar, if not

identical, in amino acid composition to the microfibrillar protein of the intact mature elastic fiber (7).

## DISCUSSION

### *Aortic Cells in Culture*

Several investigators have studied the ability of cells to grow from explants of aortic tissue (1–3, 9–11). Most of the studies examined the cells by light microscopy and described them as “fibro-

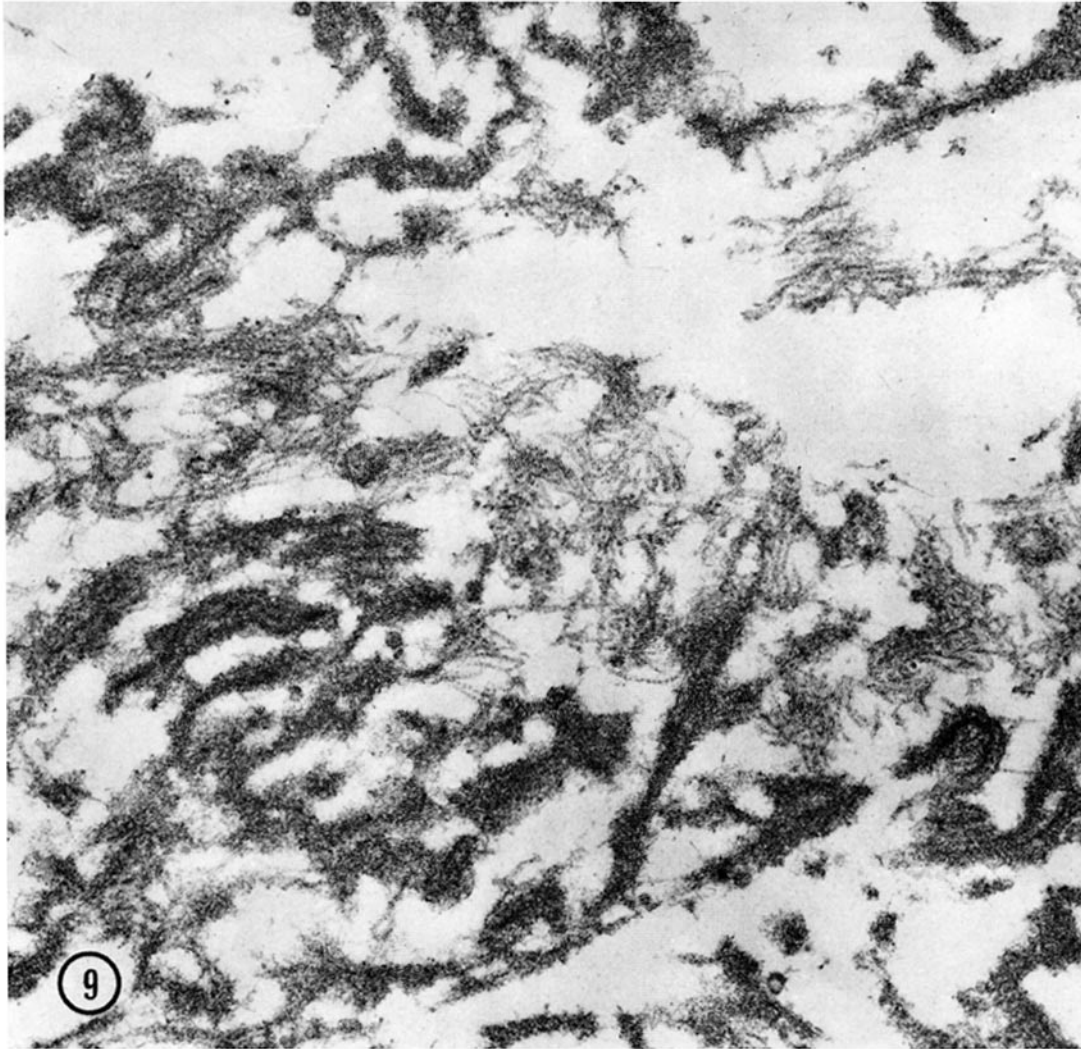


FIGURE 9 This micrograph demonstrates the material seen in the extracellular space of a 4-wk-old culture. Large amounts of amorphous, somewhat dense material similar to basement membrane material are present, at this time, together with numerous 100 A microfibrils.  $\times 40,000$ .

blast-like" (9-11), smooth muscle, or "ribbon-like cells" (2), or as mixed populations containing fibroblasts, endothelium, macrophages, and smooth muscle (1, 3). Jarmolych et al. (3) examined by electron microscopy the outgrowth from an explant which was derived from the middle of the media of the pig thoracic aorta. They described primitive cells, fibroblast-like cells, and modified smooth muscle cells in the outgrowth (3). In most cases, their cells contained relatively sparse myofilaments, generally located at the cell periphery. They also described the formation of

collagen, mucopolysaccharides, and elastic fibers. Since the cells that grew out of their explants represented a heterogeneous population of cells, they were unable to ascribe formation of these connective tissue components to any specific type of cell.

All of the experiments in the present studies were performed with cells obtained from the ninth subculture or later, so that no material was carried over from the original explant. The cells that formed in the present experiments represent a uniform population of cells, in terms of their

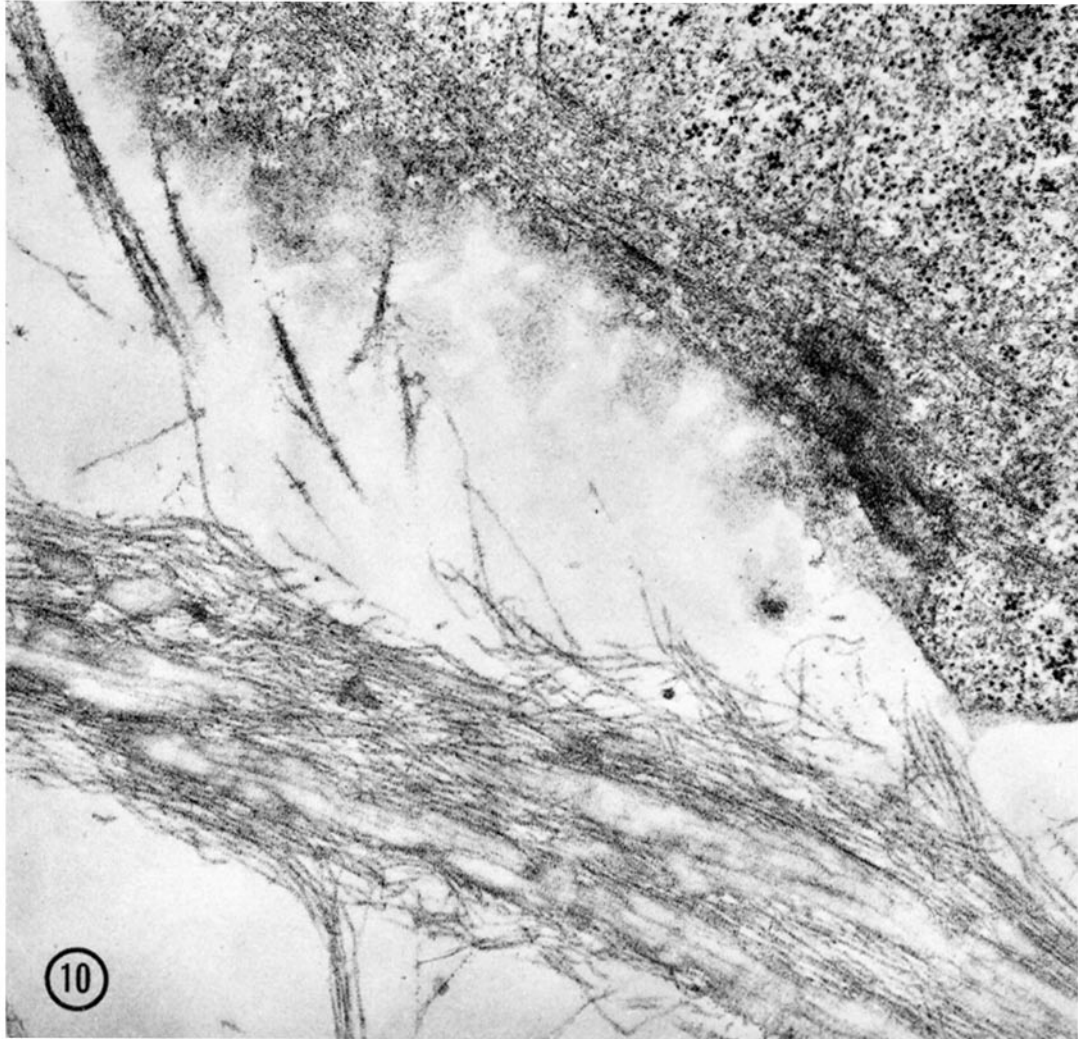


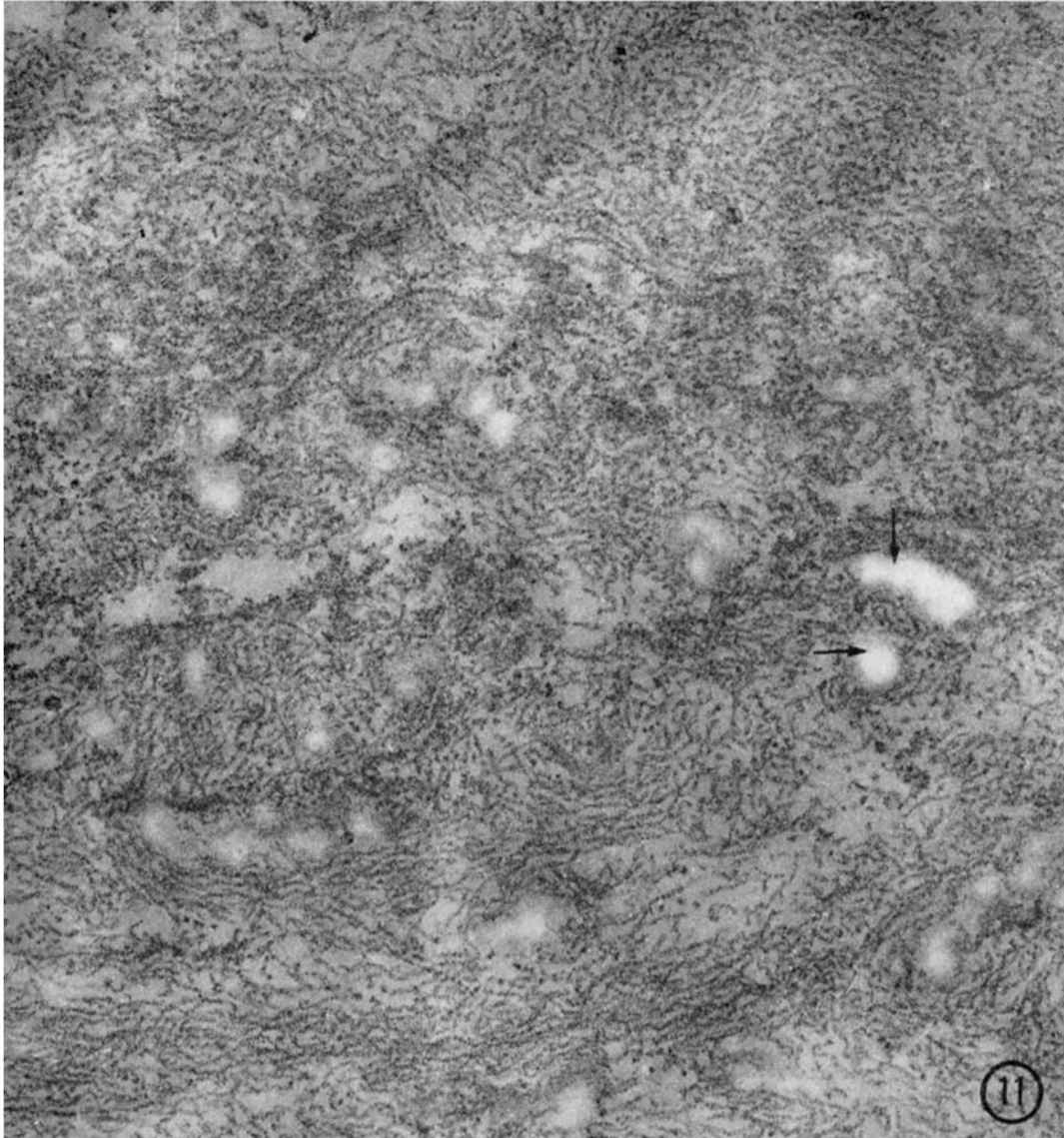
FIGURE 10 A tangential section of an 8-wk-old culture in which an aggregate of amorphous material and microfibrils, similar in appearance to those seen in developing elastic fibers in vivo, are present adjacent to one of the cells.  $\times 30,000$ .

morphology. The principal feature relating them to smooth muscle was the large volume of cytoplasm occupied by filaments, presumed to be myofilaments, and the formation of a basement membrane-like substance at the cell periphery. The cells markedly resembled smooth muscle cells in vivo that are engaged in the synthesis and secretion of extracellular proteins (12-14).

The amino acid composition of the extracellular microfibrils formed by these cells clearly demonstrated that these microfibrils are identical with the same structures now known to be an integral component of the elastic fiber (6, 7). The time of

formation of these microfibrils is interesting in that they appear in culture, before the appearance of any amorphous material, presumed to be elastin. This sequence of events is similar to that seen in elastic fiber formation in vivo during embryogenesis (6, 7, 15). It is not known that the amorphous material described in these cultures (Figs. 10, 11) is elastin, since it was not possible to obtain enough of this material for amino acid analysis; however, its morphology and staining characteristics are suggestive of the elastin seen in ligaments and blood vessels.

The growth of the cells mimics that seen in the



**FIGURE 11** This micrograph represents a pellet derived from an elastic fiber extract of 30 cultures which were harvested, homogenized, and extracted in guanidine followed by collagenase. The pellet is rich in 110 A microfibrils and also contains few amorphous components (arrows). Amino acid analyses of pellets such as this demonstrate that the microfibrils have an amino acid content similar to that described for the microfibrils extracted from intact elastic fibers.  $\times 42,000$ .

aorta, in that the cells form multiple layers, most of which are separated by basement membrane-like material and microfibrils.

Future studies should establish the nature of the basement membrane-like material as well as the material presumed to be elastin.

#### *Smooth Muscle Vs. the Fibroblast*

These in vitro observations, coupled with the radioautographic studies in vivo (19), clearly demonstrate that the smooth muscle cell plays a role in connective tissue formation as well as in contraction. It is the principal cell responsible for

the formation of the extracellular proteins of the arterial wall and ontogenetically and phylogenetically may be related to fibroblasts, osteoblasts, and chondroblasts.

Smooth muscle cells and fibroblasts demonstrate similar morphological features in culture. The principal difference between the two cells relates to their number and distribution of cytoplasmic filaments. Similar filaments are present in fibroblasts, although they are scattered throughout the cytoplasm in small bundles rather than occupying large segments of the cytoplasm as in the smooth muscle cells. These observations, coupled with the fact that smooth muscle can form connective tissue proteins *in vivo* (19) as well as *in vitro*, raise the question of the relationship between these two cell types.

Cultures such as these may provide worthwhile models for studying various aspects of atherogenesis. Several attempts were made in this direction in earlier studies (11). It is now well established that the cells involved in proliferation in the atherosclerotic plaque are smooth muscle (16-18). The ability to study these cells, particularly after cloning, under conditions which may be related to atherogenesis, may provide further understanding of the etiology of this important disease. Such cells also provide a means for a systematic study of the synthesis of elastic fiber proteins *in vitro*.

In addition, the ability to obtain intact elastic fiber microfibrils *in vitro* may permit a further analysis of their interaction with elastin precursors during elastic fiber formation.

The author would like to gratefully acknowledge the technical assistance of Mrs. Claire Hancy, Mrs. Marcia Usui, and the electron microscope laboratory of the Department of Pathology. We would also like to thank Paul Bornstein for providing the amino acid analyses.

These studies were supported in part by grants from the United States Public Health Service, AM-13970 and GM-13543.

Received for publication 17 September 1970, and in revised form 30 October 1970.

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