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Human Atherosclerotic Plaque Cells and Leiomyoma Cells

Comparison of In Vitro *Growth Characteristics*

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Cells derived from human atherosclerotic plaques and from arterial media were compared with cells obtained from human leiomyomata and myometrium with respect to growth behavior in long-term cell culture. None of numerous variations in culture media, including alterations of serum concentration and source, improved the rate of cell multiplication or *in vitro* longevity. Both uterine cell types, but neither arterial cell type, multiplied after tissue dissociation with enzymes (elastase, collagenase, hyaluronidase). The replicative life-span of each of eight samples of arterial plaque cells was equal to or less than that of the corresponding medial cells. A similar relationship was observed for eight paired sets of leiomyoma and myometrial cells. The results indicate that, under the conditions of culture *in vitro*, cells of a *bona fide* smooth muscle tumor have a finite replicative life-span and smooth muscle cells of atherosclerotic plaques behave in a similar manner. (Am J Pathol 78:175–190, 1975)

THE ROLE OF THE SMOOTH MUSCLE CELL as the principal cellular component contributing to the formation of atherosclerotic plaques is now widely recognized.[1–3] The vascular media, similarly composed of smooth muscle cells, has been thought to be the source, through proliferation and migration, of the population of smooth muscle plaque cells,[4–6] although this origin of the plaque cells has not been clearly

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demonstrated. Nevertheless, the capability of the plaque cell to form progressively enlarging, apparently physiologically functionless tissue masses composed primarily of a single cell type suggests that the atherosclerotic plaque can be viewed as a kind of benign smooth muscle tumor. The relationship between plaque and media may then be analogous to the relationship of a traditionally recognized smooth muscle tumor, the uterine leiomyoma, to its parent tissue, the myometrium. This view is reinforced by the recent demonstration that human atherosclerotic plaques, like human uterine leiomyomata, are monoclonal in origin.[7] The known tendency for both to become fibrotic and calcified further heightens the analogy.

The present work was designed to explore further the relationships between the two pairs of tissues, plaque and media, and leiomyoma and myometrium. Since the capacity to form tumorous growths is a characteristic shared by both leiomyoma and plaque and since little is known of the *in vitro* growth behavior of either plaques or benign tumors, examination and comparison of the growth characteristics of these tissue types with respect to their normal counterparts in cell culture seemed a reasonable place to begin. In addition, the growth characteristics of a third smooth muscle of unrelated source, human duodenal muscularis, were examined for added comparative purposes.

To encourage the greatest possible outgrowth from the tissue explants and the maximum expression of differences in growth rates and growth patterns, the influence of various combinations of culture media and sera was investigated. Finally, in an effort to reduce the artifacts of tissue sampling, with inequalities of starting sample size and limitations of cell number, certain of the samples were subjected to enzymatic digestion of the extracellular matrix to obtain dissociated cells representing, as nearly as possible, the entire sample. The cells from these samples could then be plated in equal known numbers, providing better estimates of population increases.

Materials and Methods

Tissues

All of the specimens listed in Tables 1 and 2 were obtained at the time of surgery. The samples of femoral artery were obtained in each instance from an extremity amputated because of the sequelae of arterial insufficiency. The uteri and their leiomyomata were obtained from hysterectomies performed for such conditions as vaginal bleeding or pelvic relaxation. A single specimen of human duodenal muscularis removed at surgery was diced and explanted. Additional samples of aorta and femoral arterial tissue from autopsy, as well as surgical material and

Table 1—Replicative Life Span of Human Femoral Artery Cells

Patient			No. of population doublings		Weeks of active doubling	
Age (yrs)	Sex	Diabetes	Media	Plaque	Media	Plaque
46	M	+	0	1	0	4
56	M	+	8	2	7	3
58	M	+	16	0	22	0
63	M	—	10	9	10	10
64	M	+	3	3	4	3
66	M	+	4	4	5	16
73	M	+	15	14	36	56
81	F	—	11	5	12	12

the cells derived from outgrowths of these samples, were used in the evaluation of the media and sera described below.

Media and Sera

The following media were evaluated: Waymouth's (prepared in the laboratory of Dr. George M. Martin, University of Washington), the Dulbecco-Vogt modification of Eagle's basal medium [8] (Dr. George M. Martin) and Ham's F-12 (Gibco, Berkeley, Calif). The following supplemental sera (Microbiological Associates, Albany, Calif) were used individually or mixed together in various proportions to yield a final total serum concentration of 10 or 20%: fetal bovine, human adult or human cord. These sera were employed variously in one or another of the above media. All media were enriched as well with added nonessential amino acids. "Conditioned medium"—Dulbecco-Vogt medium containing 10 or 20% fetal bovine serum "conditioned" by having supported cell growth for several (usually 3) days—was also used. Table 3 lists the different combinations of sera and media evaluated and the total number of cases in which each was used.

Table 2—Replicative Life Span of Human Uterine Cells

Patient age (yrs)	No. of population doublings		Weeks of active doubling	
	Myometrium	Leiomyoma	Myometrium	Leiomyoma
32	14*	5	42	22
43	6	2	31	13
43	17*	9	41	34
48	24	6	29	11
49	39*	0	40	11
52	9*	2	32	23
56	11	2	23	11
59	1	0	18	0

* Current lines

Table 3—Composition of Culture Media

Basic medium	Added serum	Percent serum	Cases
D-V	NBC	10	7
D-V	NBC	20	17
D-V	FB	10	11
D-V	FB	20	17
D-V	C	10	7
D-V	H	10	8
D-V	C + FB	5 + 5	9
D-V	H + FB	5 + 5	2
F-12	FB	10	2
Way	NBC	10	6
Way	NBC	20	6
Way	FB	10	5
Way	FB	20	6

D-V = Dulbecco-Vogt modification of Eagle's medium, F-12 = Ham's F-12, Way = Waymouth's medium, NBC = newborn calf serum, FB = fetal bovine serum, C = human cord serum, H = adult human serum.

Explanting

Upon retrieval from the operating room, the specimen of artery was cut into longitudinal strips. With the use of fine forceps a plane of cleavage was then carefully developed between the intima, often eccentrically thickened, and the circularly oriented fibers of the media (Figure 1). The inner intimal layer was removed, and the thickened portion was diced into 1-mm cubes. Four cubes were randomly selected and placed into each of eight Leighton tubes. One milliliter of the particular combination of medium and serum under investigation was then placed in the tubes. A fitted glass slide was put into each tube to "sandwich" the explants between slide and tube; the tubes were closed and placed in an incubator at 37 C containing a constant gas mixture of air and 5% CO_2. The media was peeled from the remaining outer layers of media-adventitia in circumferential strips. These were diced and similarly explanted. On occasion a definite plane of cleavage could not be established between intima and media. In that case the piece was not used. The tubes were left undisturbed for about 10 days, at which time semiweekly feedings were begun. After 3 or 4 weeks the coverslips with attached explants were removed from the Leighton tubes and transferred to 25-cm Falcon flasks, where feeding was continued twice per week. (Somewhat greater cell yield has more recently been achieved by explanting 20 tissue cubes directly into 25-cm Falcon tissue culture flasks containing 1 ml of medium.)

With regard to the uterine specimens, generous pieces of well-circumscribed, firm, nondegenerated leiomyomata were dissected from the removed uterus in the operating room. Care was taken to avoid including any portion of adjacent myometrium. Similarly, samples of the myometrium were taken and placed in a container separate from the myoma, and both were removed to the laboratory. There each tissue was diced using separate sets of instruments and explanted as above. The sample of duodenal muscularis was similarly handled.

Enzymatic Dissociation

In addition to explanting, when the specimen was large enough (as was not always the case with samples of artery), the tissue was more finely minced with

scalpel blades. About 2 g of the minced tissue was placed in a 25-ml weighing bottle and covered with about 8 ml of Dulbecco-Vogt medium containing 400 units/ml crude collagenase (Worthington Biochemical Corp, Freehold, NJ), 320 units/ml hyaluronidase (Worthington) and, in the case of arterial specimens, 0.2 mg units/ml elastase (Sigma). This material was then slowly stirred at room temperature for up to 3 hours or until the majority of tissue fragments disappeared. The mixture was then filtered through nylon mesh, the filtrate centrifuged (1,000 rev/min for 10 minutes), and the pellet washed with fresh medium. Centrifugation and washing were repeated twice, and the cells were then suspended in the chosen serum-containing culture medium. The concentration of arterial cells in the suspension depended on the amount of tissue originally available and was often in the neighborhood of 5×10^4/ml. Uterine cells were plated at a concentration of 2×10^5/ml. Two milliliters of the suspension were placed into 25-cm Falcon flasks and incubated as above. A drop of each cell suspension was removed, mixed with one drop of nigrosin [9] and examined for dye exclusion. After standing for 72 hours to allow for cell attachment to the flask bottom, the supernatant medium containing debris and unattached cells was decanted. Two milliliters of fresh culture medium were added, and the flasks were then fed regularly twice a week.

Subculturing

When the cell number (either as outgrowth from tissue explants or as descendants of a primary plating of dissociated cells) increased to form a confluent layer over all or most of the flask bottom or when the cell layer threatened to peel off the surface, the cells were removed from the flask by trypsinization (0.5 mg/ml for 10 minutes) and were counted and replated at a concentration of 8×10^4 (arterial cells) or 4×10^5 (uterine cells) per 25-cm flask. This first subcultured flask with its known population of cells was used as the starting point in calculating the rate of population growth.

Microscopy

In all cases, representative portions of unprocessed tissue were reserved for both light and electron microscopy. In a few cases tissue samples were retrieved for electron microscopy following partial enzymatic digestion. In addition, histologic preparations were made of representative cell outgrowths and subcultured populations. The cells were generally fixed in alcohol-acetic acid and stained with toluidine blue or hematoxylin and eosin. At intervals in their growth, cell samples were also embedded in Epon for electron microscopy.

Results

Media and Sera

No combination of medium and serum was found to be any more effective in encouraging outgrowth from the explants or multiplication of subcultured arterial cells than Dulbecco-Vogt containing 10 or 20% fetal bovine serum. Moreover, neither of the cell populations derived from media or plaque displayed any individual growth rate response to medium changes not shown by the other. No particular combination of medium and serum produced consistent morphologic changes in cells

or clones. It was observed, however, that undiluted conditioned medium provided the best environment for freshly trypsinized cells. Generally, a plating efficiency of 90% or better could be achieved within 48 to 72 hours following trypsinization. Cells dissociated from tissue samples of myometrium or leiomyoma placed in conditioned medium diluted three times with fresh medium tended to settle more rapidly and yield a higher plating efficiency than in fresh medium alone.

Enzymatic Dissociation

When minced samples of myometrium and leiomyoma were exposed to enzyme mixtures of collagenase and hyaluronidase for up to 3 hours, they yielded large numbers of cells (averaging about 50×10^6 cells/2 g of tissue) of which about 70% excluded nigrosin. When plated in Dulbecco-Vogt with 10% fetal bovine serum, supplemented with 25% conditioned medium, approximately 50% settled within 3 days and commenced to multiply. When minced samples of artery (plaque or media) were similarly treated (or treated with enzyme mixtures of collagenase, hyaluronidase and elastase), cells (up to 6×10^5) were obtained which (in 11 of 11 attempts) failed to settle in significant number, remained rounded, and eventually degenerated despite having an initial viability (by dye exclusion) of 70%. When cell layers derived from outgrowths of arterial explants were similarly treated with mixtures of collagenase, hyaluronidase and elastase, minimal cytotoxicity was demonstrated and the cells settled with a plating efficiency only slightly less than cells trypsinized in routine subculturing.

Replicative Life Span

In Table 1 are shown the results of long-term cultures of eight femoral artery specimens maintained in Dulbecco-Vogt medium containing 20% fetal bovine serum. In these cases the separation between plaque and media was felt to be reliable, and both types of tissue yielded sufficient numbers of cells in the outgrowth to be subcultured at least once. Following a mean period of 5.6 weeks (SD, ± 0.7), the Leighton tubes containing explants of arterial media yielded a mean of 9.8×10^4 (SD, $\pm 2.9 \times 10^4$) cells per tube. A mean of 5.6×10^4 (SD, $\pm 1.2 \times 10^4$) plaque cells was harvested from each tube after a mean of 8.0 (SD, ± 1.2) weeks. As can be seen from Table 1, in all cases the number of population doublings was finite and varied from 0 to 16, while the period of active doubling varied from 0 to 56 weeks, although some populations could be retained in a viable but nonincreasing state for up to 100 weeks. In all cases but one, the total number of doublings of the

medial cells was the same as, or greater than, the doublings of the plaque cells.

In Table 2 are shown the results of long-term cultures of eight paired specimens of myometrium and companion leiomyoma. In each case the cells were obtained by enzymatic dissociation of the tissue and were maintained in Dulbecco-Vogt with 10% fetal bovine serum. In all eight cases the cell lines derived from the leiomyomata have been discontinued because of cessation of growth after achieving zero to nine total population doublings. Four of the myometrial cell lines are still actively growing, one of them after 39 doublings in 40 weeks. In every pair, the number of doublings accumulated by the myometrial cells was greater than the number of doublings accumulated by the leiomyoma cells. For comparison, we examined the long-term growth of a specimen of human duodenal muscularis. As with the uterine specimens, the cultures were begun from a washed pellet of dissociated cells derived from the tissue by digestion with collagenase and hyaluronidase. The cell line achieved a total of 20.4 population doublings in 33.5 weeks, after which the cell population ceased to increase and the line was discontinued.

Morphology

In general, the early outgrowth from explants of arterial media and plaque developed as densely cellular parallel arrays of slender spindle-shaped cells that were frequently multilayered, with the layers running at angles to each other (Figure 2). Often, with further development, the periphery of the outgrowth tended to show clustering of cells into more tightly arranged groups, while slender more loosely arranged cells connected groups in a radial fashion (Figure 3). Associated with cultures in which population growth had slowed, the cell clustering frequently became striking, with the cells aggregating into rounded, structureless masses which stained intensely with toluidine blue and frequently became detached from the flask. Electron microscopically, these masses were seen to consist of granular debris containing cell fragments and occasional intact cells and to be lined on the surface with cells that appeared viable (Figure 4). The development of detaching and of rounded aggregations was common to cultures derived from plaque and medial tissue but was rare in cultures of myometrium, myoma, or intestinal samples, although the pattern of loose and dense cellular arrays was common to all cultures. Several histologic and cytologic features were common to cultures marked by slowed or absent population increases regardless of tissue source. These included a loosening of the cellular texture, with loss of the parallel array of cells, and

a change in the outlines of many cells from slender and bipolar to polygonal or even dendritic, accompanied by cell enlargement and the appearance of bi- and multinucleate forms (Figure 5). Ultrastructurally, cells from these cultures often showed features of degeneration characterized by increasing cytoplasmic myelin figures and the cytoplasmic accumulation of large numbers of lysosome-like bodies filled with amorphous, finely granular material (Figure 6).

Typically, the cells examined ultrastructurally from an active culture from whatever source showed to varying degrees the features characteristic of smooth muscle: tightly packed myofilaments, dense bodies, and peripheral vacuoles (Figures 7 and 8). Only very rarely was some extracellular material that might resemble basal lamina seen juxtaposed to a plasma membrane. In cultures of actively growing muscle cells one could not distinguish the tissue origin of the cultures, either by growth pattern or cytologically, using light and electron microscopy. In no case, either recently established or in late passage, were cells observed that possessed the great abundance of myofilaments and dense bodies characteristic of smooth muscle cells *in vivo*. Frequently, in cultures of any chronologic age in the laboratory, cells could be found lacking any smooth muscle features adjacent to cells that had clearly recognizable features of muscle cells. In older cultures the proportion of such "featureless" cells was higher than in recently derived cultures.

Discussion

To draw an analogy between the arterial plaque and the uterine leiomyoma is useful only if it stimulates inquiry into the fundamental property shared by both, namely the capacity to engage in apparently physiologically functionless cell proliferation. With regard to the plaque, the proliferation has been viewed as a reaction to injury either directly to the medial smooth muscle [5,6,10] or indirectly via altered permeability of an injured overlying endothelium.[11] No similar argument has been advanced to account for the initiation and growth of the leiomyoma.

The principal observation of this study is that cells derived from uterine leiomyomas and arterial plaques have a finite replicative lifespan and population doubling rate *in vitro* that are at least as short as the life-span and doubling rate of cells derived from the parent tissues. In particular, in all 8 cases studied the leiomyoma cells were found to be distinctly shorter lived than the companion myometrium, whereas this was true of only 3 of 8 cases of arterial origin. The study also shows the sluggishness of growth of human adult smooth muscle cells (averaging considerably less than one doubling per week) and the relatively

few doublings the arterial cell populations engage in before becoming stationary. Furthermore, the arterial tissues, when compared with uterine or intestinal muscle, were much more vulnerable to the toxic effects of enzymatic dissociation since they did not yield cells in any number that would settle and grow, while the uterus and intestine regularly did. Arterial cells (once established in culture) do not seem to be seriously affected by the enzymatic treatment that seems irreversibly to damage the cells during removal from tissues. This suggests either selection of a cell type which is resistant to such treatment or the "conditioning" of cells by cultivation and transfer.

Electron microscopically, the cultured cells from all groups showed to some degree the features permitting their identification as smooth muscle and, despite the behavioral differences noted above, were morphologically indistinguishable from one group to the next. The progressive morphologic changes undergone by all of our cells in long-term culture, including increasing size and the intracellular accumulation of numerous lysosome-like bodies, have been reported by others studying cells from several sources and are considered now to be indicators of *in vitro* senescence.[14,15]

In previous work [12] we have emphasized the differences between plaque cells and medial cells that can be observed *in vivo*. In studies on the spontaneously occurring aortic plaque in the chicken, it was observed that plaque cells are smaller in size, are oriented longitudinally rather than circularly, possess few intercellular junctions and are responsible for the local elaboration of an abundant collagen in the plaque rather than elastin as found in the media. In addition, plaque cells show a striking response to cholesterol feeding not manifested by the underlying media.[13] Therefore, we felt it was reasonable to attempt to look for differences between these two cell populations *in vitro* where, if found, they could be more closely analyzed. The present study did not yield appreciable differences: the cells of the plaque and of the media remain indistinguishable morphologically and behaviorally despite numerous manipulations of growth conditions. Similarly, although the leiomyoma *in vivo* displays obvious architectural differences from the surrounding myometrium and its increased collagen content can be appreciated grossly, no morphologic differences from the myometrium were observed or induced *in vitro*. A comparison of some metabolic capabilities among the four cell types is currently under investigation.

Finally, the finding that a true benign tumor, the leiomyoma, has a finite replicative life span *in vitro* and multiplies to a lesser extent than the myometrium may seem paradoxical, since one might assume that

tumors should have some replicative advantage over normal somatic cells. It has been reported, for example,[16,17] that vigorous outgrowths of cells may be obtained from explants of cervical carcinoma, while explants from adjacent normal cervical epithelium do poorly. Similarly, cells transformed by viruses multiply rapidly *in vitro* and apparently acquire the property of immortality in culture.[18–20] However, in an interesting series of studies, Rous and Kidd[21] observed and emphasized the "conditional" nature of tumors induced by tar painting or by purified carcinogens. They point out that in the benign tumors,

> . . . the neoplastic condition gives the cells a superiority over their neighbors when both are admitted to the same *encouraging* influences. . . . Their state entails such disabilities, though, that they are unable to maintain themselves under ordinary circumstances, and consequently growths composed of them (tumor cells) disappear when no longer aided. . . . It is plain that the neoplastic state does not necessarily connote independence of behavior or success in tumor function. On the contrary, it may render cells unable to survive or endow them with powers which they can exert only under favoring conditions.

It seems that we have not found thus far the "favoring" conditions permitting, in cell culture, the leiomyoma cells to exhibit their selective advantage.

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[*Illustrations follow*]

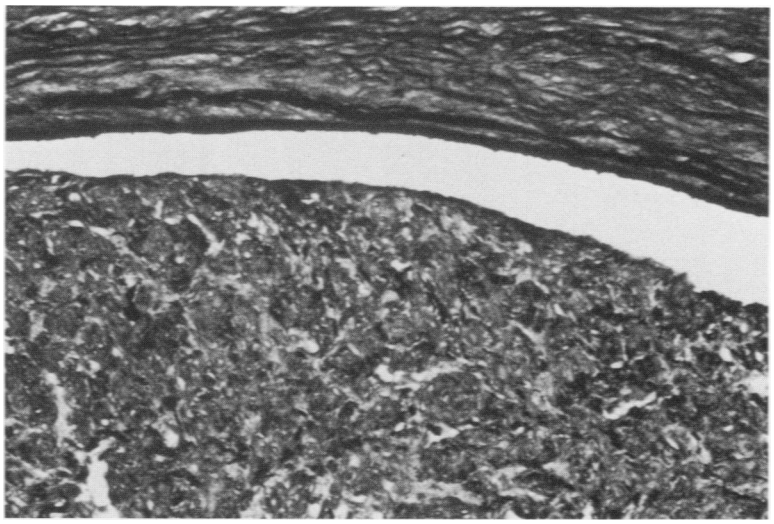


Fig 1—Longitudinal section through femoral artery obtained at autopsy in a 64-year-old male. Note the plane of cleavage developed between the plaque (above) and the media with, in this instance, the internal elastic lamina adherent to the lower border of the plaque (Verhof-Van Gieson, × 150).

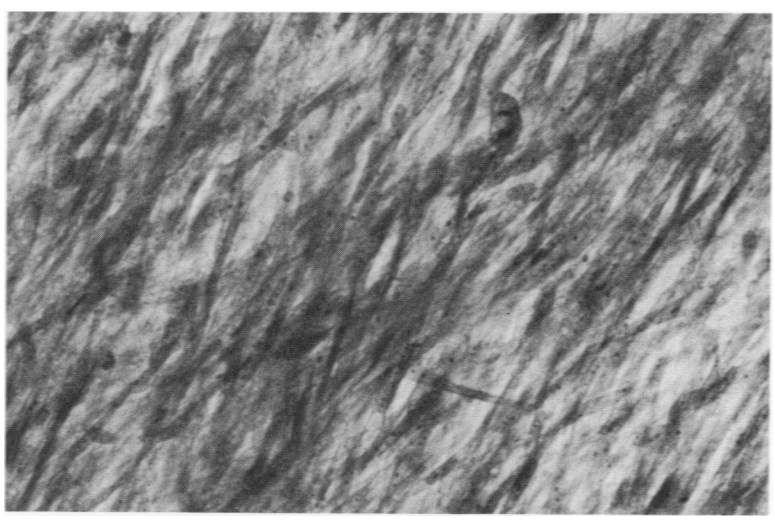


Fig 2—Outgrowth from an explant of arterial media obtained from a surgical specimen from a 73-year-old male diabetic. Note the closely packed, spindle-shaped cells (Toluidine blue, × 150).

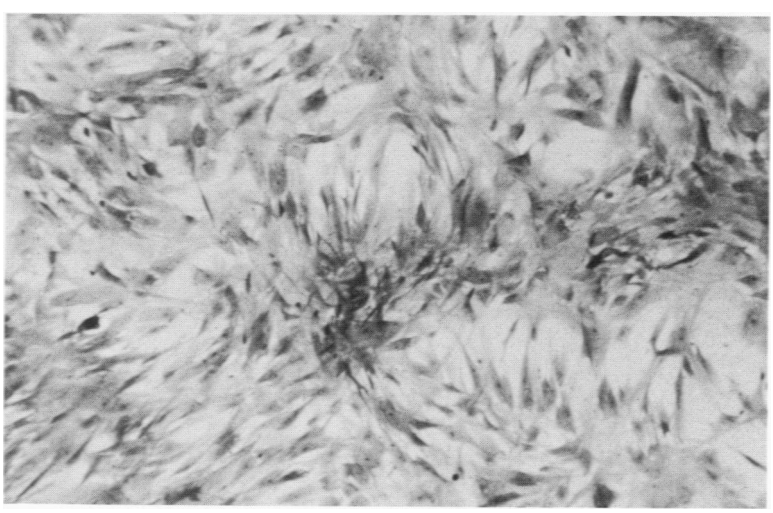
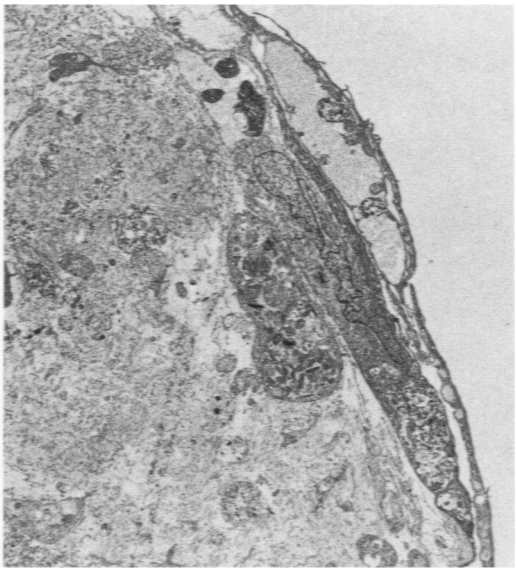


Fig 3—First passage of arterial medial cells obtained from a surgical specimen from an 81-year-old female. Note the characteristic radial arrangement (Toluidine blue, × 150).

Fig 4—Portion of an aggregate of medial cells from the seventh passage. Specimen was from a surgically amputated leg of an 80-year-old male. Note the amorphous granular debris lined by featureless cells on the surface (× 2000).

Fig 5—A large polygonal multinucleate medial cell from the same culture flask depicted in Figure 3 (Toluidine blue, × 375).

Fig 6—Two medial cells in the fifth passage from a surgical specimen of femoral artery in a 12-year-old female. Note the thin peripheral rim of myofilaments as well as the numerous cytoplasmic lysosome-like bodies (× 3200).



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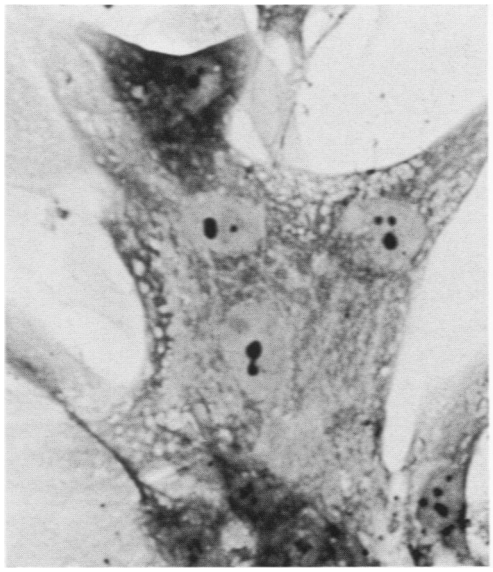

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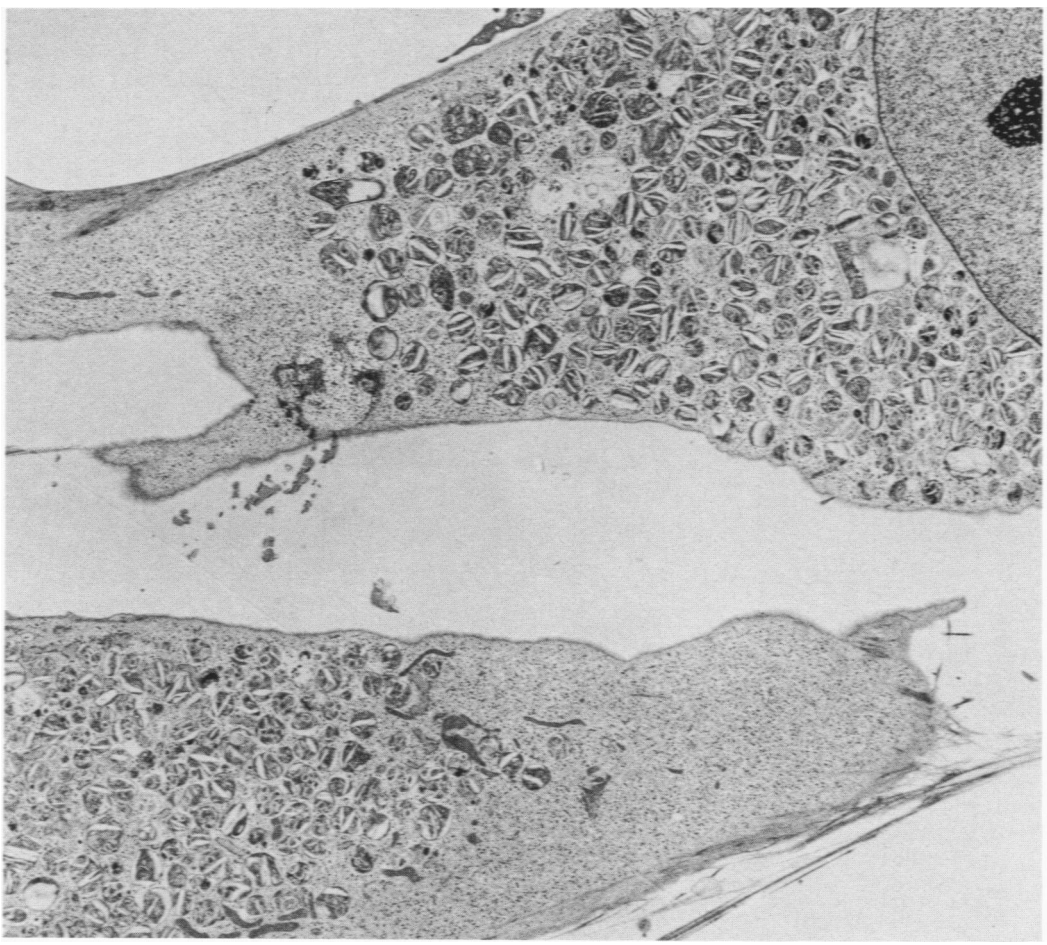

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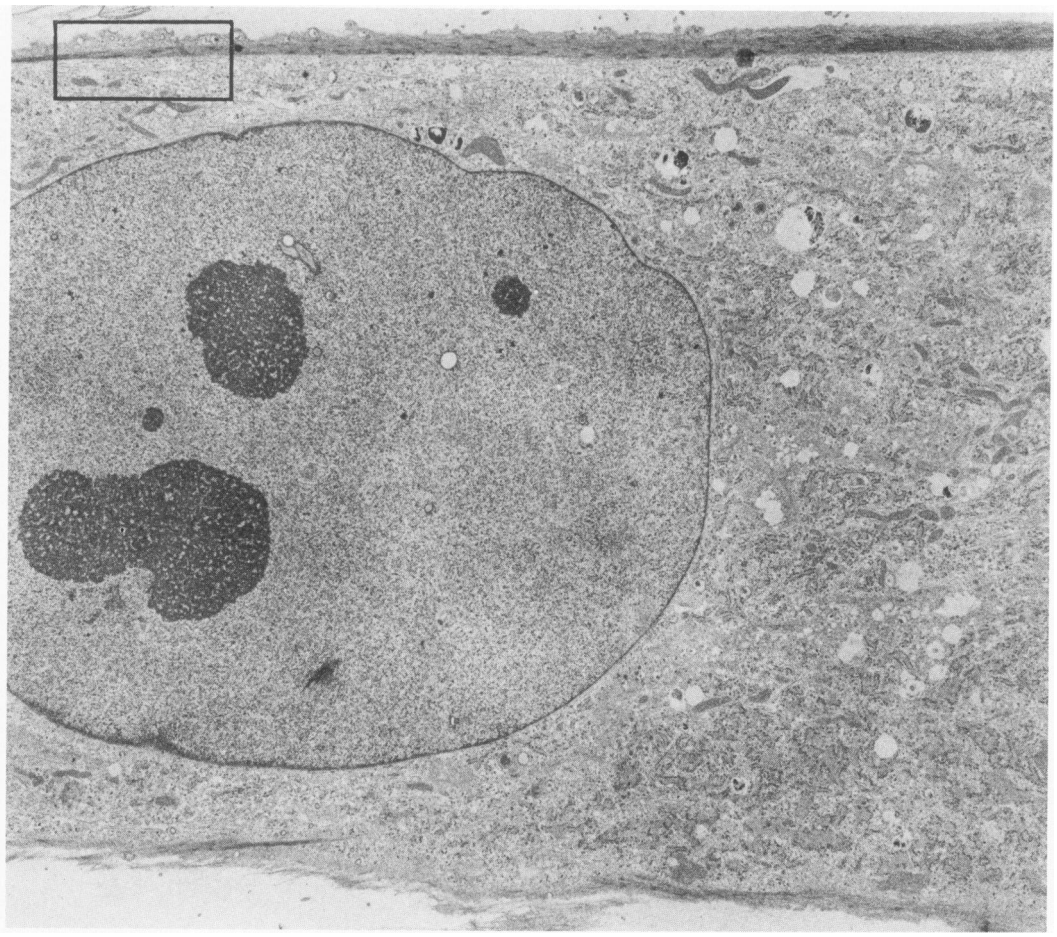

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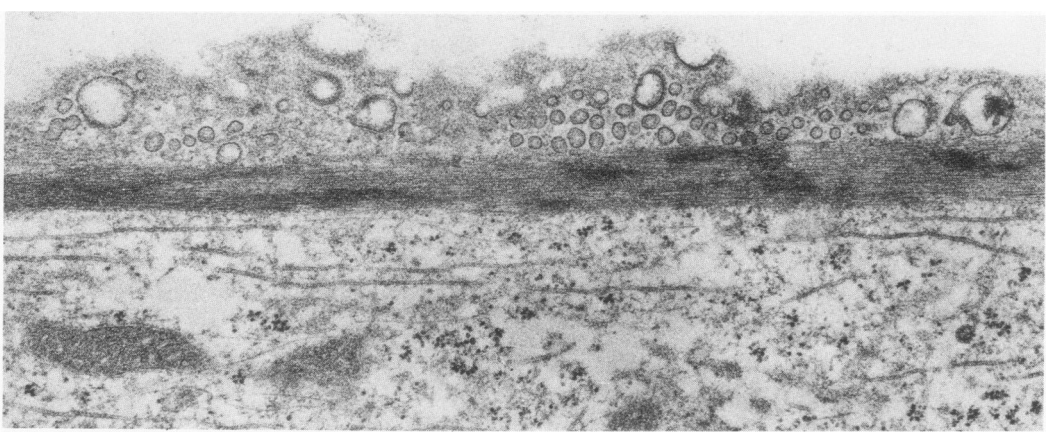

Fig 7—Outgrowth of cells derived from a plaque obtained from a surgical specimen of femoral artery in a 73-year-old male. Note the presence of a rim of cytoplasmic myofilaments (× 3600). **Fig 8**—Enlargement of boxed area in Figure 7. Note the characteristic peripheral vacuoles and myofilaments helping to identify this cell as probably smooth muscle (× 25,000).