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Comparative Phagocytosis in Culture of Aortic Smooth Muscle Cells and Fibroblasts from Rat[1] (41458)

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Abstract. Phagocytosis was studied in secondary cultures of aortic smooth muscle cells (SMC) and fibroblasts from rats. These cells were cultured with colloidal carbon particles (≃ 250 Å diameter) and the uptake was followed in living cells by phase contrast microscopy. By electron microscopy, clusters of ingested particles limited by a membrane were observed in the vicinity of lysosomes and lipid droplets. By means of quantitative analysis, it was shown that the uptake of carbon particles was 2.5 times higher in SMC than in fibroblasts. Thus in subcultures, SMC can be readily differentiated from fibroblasts by phagocytic properties. These results also indicate that SMC from rat exhibit similar phagocytic properties to those described in other animal species.

Phagocytosis is a cellular mechanism which plays a role in the uptake of macromolecules or other components. This phenomenon could be involved in atherogenesis, more specifically in the LDL uptake (1).

In that connection, phagocytosis has been described: *in vivo* for aortic smooth muscle cells (SMC) from atherosclerotic turkeys (2) and for arterial endothelial cells in different species (3); *in vitro* for SMC from pig aorta, but in the contractile state (4).

However, it seems that the cells from atheroma present some features of SMC modulated in a synthetic phenotype. Consequently, it might be of interest to investigate the phagocytic properties of SMC in their synthetic state. Such a study has been performed as described hereafter. For this purpose, secondary cultures from rat thoracic media were used; secondary cells are known to be in the synthetic state (5). Their reactivity was compared to that of fibroblasts in order to assume if the phagocytic capability of the modulated SMC were related to their muscle origin or were unspecific.

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The experiments were performed using colloidal carbon as a marker to visualize phagocytosis.

Materials and Methods. Rat arterial smooth muscle cells (SMC) and rat skin fibroblasts were used in this study.

Arterial SMC were obtained by the method developed by Ross (6) and adapted for the rat (7). The thoracic aorta was aseptically removed from 6- to 8-week-old rats (Sprague–Dawley strain) purchased from a local breeder. The adventitial layer was carefully removed and the aorta cut into rings which were transferred into the flasks. The rings were cultured in MEM (minimum essential medium, Gibco, Glasgow, Scotland) + 10% calf serum and in a 37°, 5% CO_2 humidified air environment. The culture medium was changed every 3 days. By 3 weeks, peripheral growth decreased and the cells were subcultured into flasks following trypsinization (0.08% trypsin in PBS [phosphate-buffered saline] without Ca^{2+} or Mg^{2+}).

Fibroblasts were obtained from rat skin explants and cultured under similar conditions.

Experimental procedure. Experiments were performed with cells in passage 3 to 8 seeded in petri dishes (35-mm diameter) at a density of 8000 cells per dish which allows both an easy observation and a sufficient growth (8).

Cultures were overlaid with 1.5 ml of

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medium (MEM + 10% calf serum). Two days later, the medium was renewed and colloidal carbon (China Ink Pelikan) added at a concentration of 100 μg/ml of medium. Cells were cultured under these conditions for 4 days.

Microscopy. Living cultured cells were routinely examined under a phase-contrast microscope. Cultures for electron microscope examination were treated as previously described (7). They were rinsed with PBS, fixed by 1.7% glutaraldehyde, washed in buffer, and postfixed in 2% OsO_4 (pH 7.4 in 0.1 *M* cacodylate buffer). Following dehydration, cells were embedded in Epon 812. Thin sections were lightly stained for 30 sec with lead and observed with a Phillips EM 300 electron microscope.

Quantitative measurements. The cultures were rinsed with PBS to remove all the nonphagocyted carbon particles. Then, they were fixed with a methanol–acetic acid mixture (3:1, v/v), but not stained.

Quantitative measurements were performed in an image analyzer (Quantimet 720, Cambridge Instruments), the cells being projected on a TV screen. For each cell examined at random, both the cell surface and the cellular surface occupied by carbon particles were measured in standardized conditions. The cell surface was determined by drawing the cellular profile with an electronic pencil. The cellular surface occupied by carbon particles was estimated by the method of the grey level. Surfaces were expressed in relative units: one point of surface:0.148 μm^2. For each cell, the ratio of the carbon surface to the cell surface was determined.

Statistical analysis. Correlation coefficients were calculated and the significance evaluated by Student's *t* test.

Results. *Microscopic observations.* Cell proliferation for both SMC and fibroblasts was similar in carbon-treated and in control cells. Incorporation of carbon particles was easily observed in living cells with light microscopy (Fig. 1). For the two cell types, incorporation began on the first day and increased progressively during the 4 days of culture. In the first 24 hr, the incorporation appeared greater in SMC than in fibroblasts.

In most of the cells, carbon particles accumulated around the nucleus. As shown by electron microscopy, particles of approximately the same size (100 to 250 Å) were arranged in clusters of numerous units, only a few being isolated. These clusters correspond to the carbon units observed by light microscopy. The clusters comprised approximately 20 to 300 particles in SMC (Fig. 2A) and 10 to 100 in fibroblasts (Fig. 2B). Thus in the SMC there were more clusters composed of more carbon particles as compared to the fibroblasts.

The clusters appeared limited by a unit membrane (Fig. 3). Inside the cluster, carbon particles were surrounded by a low electron density substance, and occasionally, by vesicles or myelinated membranes (Figs. 3, 4). In both cell types, secondary lysosomes and lipid droplets were often seen in the vicinity of the carbon clusters (Fig. 4).

Quantitative studies. Figure 5 shows the cytoplasmic surface occupied by carbon particles as measured by light microscopy, plotted against the surface of the corresponding cell. For the two cell types, linear regression has been calculated. The correlation coefficients are both significantly different from zero ($P < 0.001$). For SMC and fibroblasts, the amount of carbon particles incorporated by a cell increased with the cellular size. As shown by the linear model of incorporation, no saturation occurred during the experiment.

Using a *t* test, the slopes of the two regression lines were observed to be significantly different ($P < 0.001$). Therefore for a given cellular size, the phagocytic ability of a SMC is much higher than that of a fibroblast.

The ratio of the cytoplasmic surface filled up with carbon particles to the cellular surface was calculated for each cell. Figure 6 shows the distribution of the frequency of this ratio in SMC and in fibroblast cultures. For the two cultures, the distribution is unimodal; thus, SMC and fibroblast cultures might be both composed of only one major population of cells with regard to their phagocytic abilities. The means of the two distributions of the ratio: 7.47 for SMC

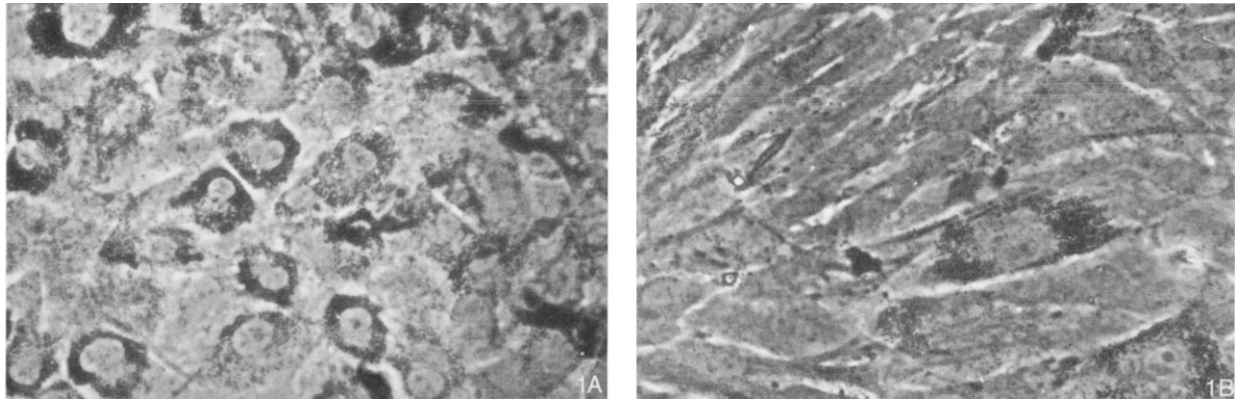

FIG. 1. Comparison of living cultures of SMC (A) and fibroblasts (B) observed with phase-contrast microscopy. Cultures have been incubated 4 days with colloïdal carbon particles. It is obvious that there are more carbon particles in SMC than in fibroblasts (×1660).

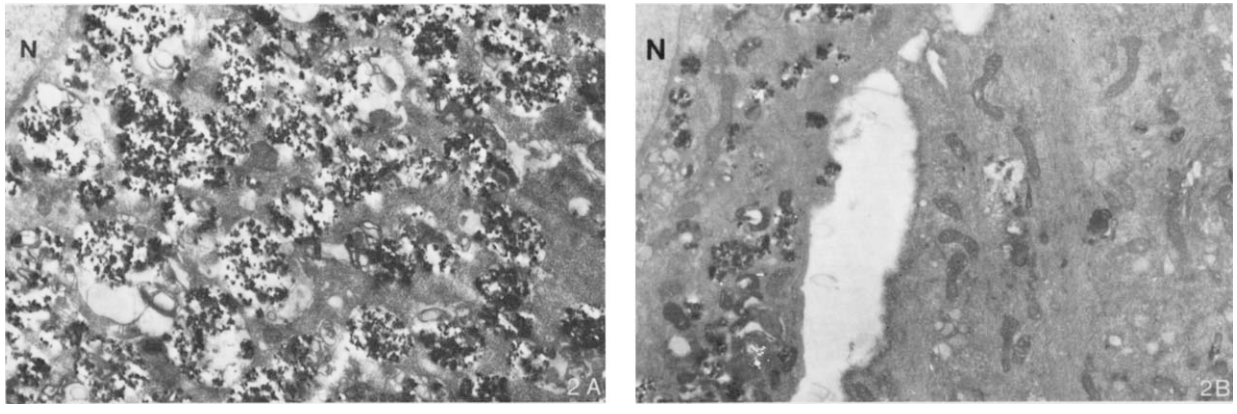

FIG. 2. Electron micrographs of a SMC (A) and a fibroblast (B) after a 4-day incubation period with colloïdal carbon. Carbon particles arranged in clusters near the nucleus (N) can be observed. In the SMC (A), as compared to the fibroblast (B), more clusters composed of more particles were noted (A) ×25,380; (B) ×20,160.

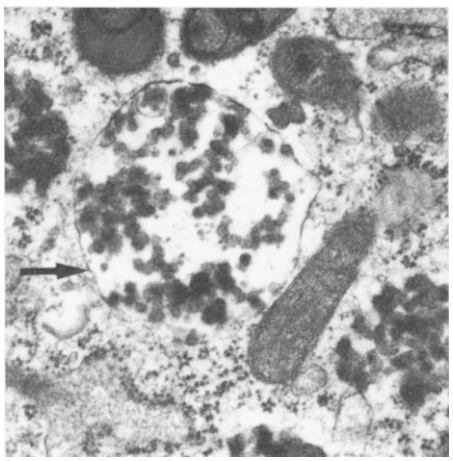

FIG. 3. Electron micrograph of a portion of SMC showing a cluster of carbon particles encapsulated by a unit membrane (arrow) (×41,800).

and 2.93 for fibroblasts were significantly different ($P < 0.001$); this is in agreement with the observation reported in Fig. 5, according to which the SMC have a higher phagocytic ability than the fibroblasts.

Discussion. Cells originating from rat aortic media explants are thought to be SMC, since in mammalian arteries the cells from the media are exclusively SMC (9). These cells have been studied for their growth characteristics (10), ultrastructural features (7), ability to secrete matrix constituents (11), and susceptibility to hyperlipemic serum (12). By contrast the nature of the secondary cells obtained by subculture of media primary cultures appears to be doubtful since they have lost their ability to contract, either spontaneously or following mechanical (13) or chemical (14) stimulation. Nevertheless, several features differentiate these cells from fibroblasts: their growth pattern, structure, collagen and elastin secretion (15, 16), elastolytic activity (17), and the presence of myosin or actin as shown by anti-myosin or anti-actin (8) antibodies labeled with fluorescein, as we confirmed recently in experiments still underway.

The present results have shown that the number of phagosomes as well as the number of carbon particles per phagosome is significantly higher in SMC than in fibroblasts. This *in vitro* phagocytic ability of muscle cells has already been observed, using latex beads, in cells derived from chick embryo heart, skeletal muscle, and in SMC from guinea pig aorta in the contractile phenotype (4). Thus, phagocytosis seems to be a general property of muscle cells. The present experiments, performed with secondary cells from aortic media, show that even in the "synthetic state" (5), cultured aortic media cells keep their high phagocytic capabilities. These results also confirm that these cultured aortic cells are not fibroblasts.

The distributions of frequencies of carbon incorporation in SMC cultures or in fi-

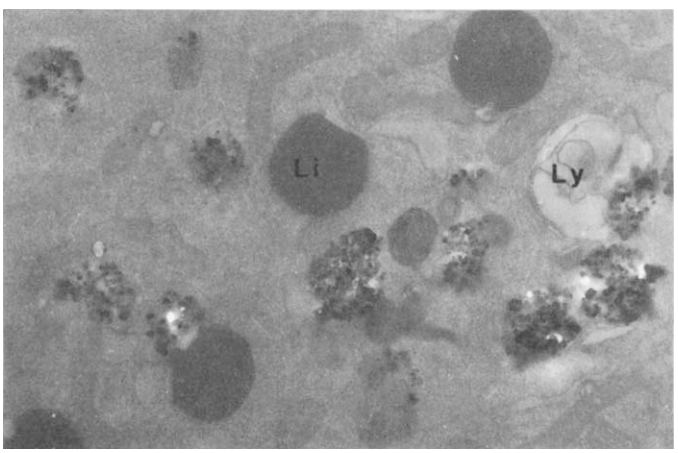

FIG. 4. Electron micrograph of a portion of SMC showing clusters of carbon particles in association with lysosomes (Ly) and lipid droplets (Li) (×106,000).

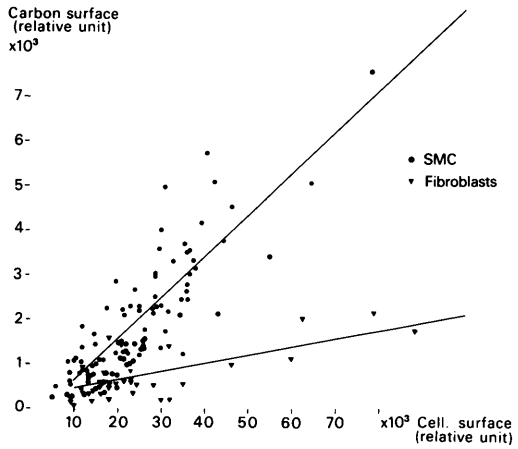

FIG. 5. Surface of ingested carbon particles as a function of the surface of the corresponding cell (SMC, slope: 0.093; fibroblasts, slope: 0.019). A population of 100 SMC in four petri dishes and a population of 34 fibroblasts also in four petri dishes were examined. The two surfaces have been calculated for each cell by means of a quantimet 720 and are expressed in relative units (one point of surface = 0.148 μm^2). The two regression lines are significantly different ($P < 0.001$).

broblast cultures is for both cell types unimodal. This suggests that SMC cultures and fibroblasts cultures consist of one cell type. Therefore, a contamination of the media cells by other cell types, either low-phagocytic cells (i.e., fibroblasts) or high-phagocytic cells (i.e., macrophages), should be, if any, very low.

From our present work and that of previous investigators (4, 19), ingested particles, consecutive to endocytosis, are incorporated into phagosomes and subsequently into lysosomes. In SMC from both guinea pig (4) and rat, as observed here, a close association between phagosomes, lysosomes, and lipid droplets was described. However, since colloidal carbon cannot be destroyed in lysosomes, the increased number of lipid droplets occurring in the cells could either be due to: (1) an increased uptake of exogenous lipids; (2) an increased supply to lysosomes of lipids such as membrane degradation products (membrane

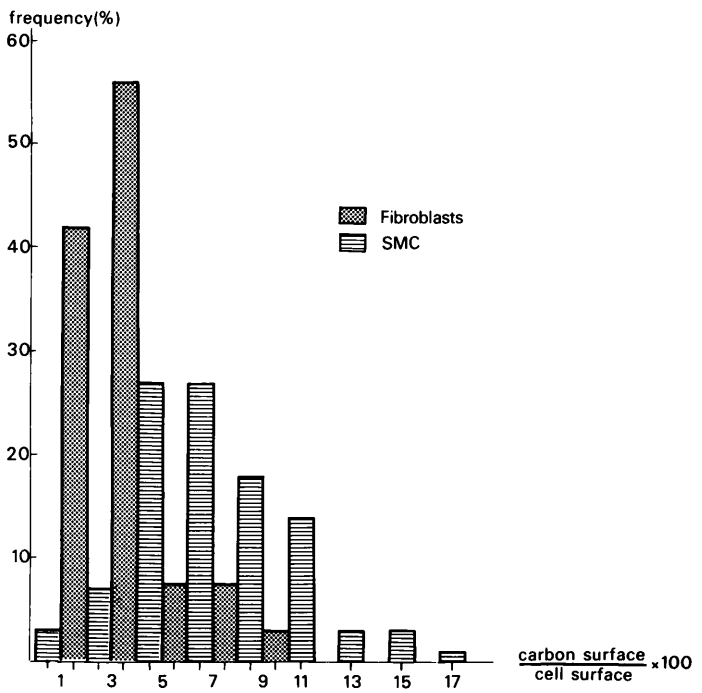

FIG. 6. Distributions of the frequencies of the ratio (surface of ingested carbon/surface of the corresponding cell) ×100. The ratio was calculated for each cell in populations of 100 SMC and of 34 fibroblasts. Note the unimodal distribution of the frequencies for both populations, SMC and fibroblasts.

turnover is enhanced by phagocytosis); or (3) an increase in cellular lipid synthesis.

Rat is well known to be rather resistant to spontaneous (20) or experimental atherosclerosis (21). The present studies indicate that rat SMC possess phagocytic properties similar to that of SMC from other species less resistant to atherosclerosis. Consequently, it seems that the resistance of rats to atherosclerosis is not due to a difference in the activity of their arterial smooth muscle cells, at least as regards phagocytosis.

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