Isolation and characterization of endothelial cells from the epididymal fat pad of the rat

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Abstract Endothelial cells from rat epididymal fat pad capillaries were isolated from rats immediately after weaning. The cells were obtained after an initial brief incubation with collagenase under conditions of minimal breakage of cells. Adipocytes were removed by flotation and endothelial cells were then obtained as cell aggregates by fractional filtration procedures whereby intact tissue as well as free cells were removed. These aggregates were then dispersed and cultured in supplemented medium 199 whereby a monolayer of cells with a growth pattern, numerous pinocytotic vesicles, and intercellular junctions typical of endothelial cells were obtained. Minor contaminations of precursor cells to adipocytes were absent after one subculture. Here >95% of the cells showed the presence of Factor VIII. Further subcultures produced nonhomogenous cells and decreasing rates of replication. The endothelial cells showed a very low rate of triglyceride synthesis and release, and collected no visible lipid upon prolonged cultures in the presence of an abundance of triglyceride substrate. They bound lipoprotein lipase from rat adipocytes, whereby the lipase was stabilized. This binding was released by heparin, and the cells did not synthesize the enzyme.—**Björntorp, P., G. K. Hansson, L. Jonasson, P. Pettersson, and G. Sypniewska.** Isolation and characterization of endothelial cells from the epididymal fat pad of the rat. *J. Lipid Res.* **24:** 105–112.

Supplementary key words adipose tissue • pinocytotic vesicles • triglyceride synthesis • triglyceride hydrolysis • lipoprotein lipase • heparin release.

Endothelial cells are strategically situated on the inner lining of blood vessels. There they exert a number of important functions in the transport processes between blood and tissues as well as in local reactions, e.g., promoting hemostasis. In order to study such cellular functions in detail it has proved valuable to use isolated cells or cells that have been isolated and then cultured. Endothelial cells have been isolated for such purposes from large blood vessels either by mechanical or enzymatic procedures (1–4). Using this type of material as a source of endothelial cells, contamination of other cell types constitutes a major risk, particularly since the rate of multiplication of endothelial cells from the adult organism often is rather slow in comparison with that of contaminating cells (5). Highly standardized procedures utilizing the bovine aorta (6), or blood vessels from the cord of the human placenta (3) minimize these technical difficulties (3), and such techniques currently seem to be the methods of choice for work with isolated endothelial cells.

Another way to diminish the mentioned contamination difficulties is to use capillaries as the source of endothelial cells. This has been done with capillaries from the brain of rats (7) and cattle (8), and from adipose tissue of rats (9). These methods are, however, not free from problems. Cells other than capillary endothelial cells occasionally contaminate the culture and subculture is difficult.

A method for the isolation of cells from the epididymal fat pad of small rats has recently been described and these cells develop on subsequent culture into adipocytes (10). In the isolation procedure another cell fraction was removed which turned out to consist mainly of capillary endothelial cells. Since such cells from adipose tissue have been suggested to be functionally integrated with the adipocytes, it was also of interest to isolate these cells. In the present work simple procedures are described that liberate these cells and allow them to be collected in such a purified form that in subsequent cultures they apparently form a homogenous population. It has also been shown that these cells bind lipoprotein lipase.

MATERIALS AND METHODS

Experimental procedures

Male Sprague-Dawley rats, 20–25 days of age, weighing approximately 40–50 g, were fed ad libitum with commercial rat pellets (EWOS, Södertälje, Sweden) con-

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taining 5, 55, and 22.5% fat, carbohydrate, and protein, respectively, plus minerals and vitamins. They were also given boiled potatoes and as many as possible, up to a number of ten, were housed in each cage at about 25°C. They were killed by exsanguination under diethyl ether anesthesia and the testis, epididymis, and the epididymal fat pad with its vessels were removed under sterile conditions and placed in a Petri dish with physiological saline. Here the epididymal fat pad was removed from the epididymis, and the vessels outside the tissue as well as the major branches within the tissue were then also removed with a pair of scissors.

Liberation of cells

Four epididymal fat pads were cut into pieces of about 25 mg and incubated to liberate the cells in 50-ml siliconized Erlenmeyer flasks containing 10 ml of a solution, pH 7.4, with final concentrations of 0.1 M HEPES buffer (hydroxyethylpiperazineethanesulfonic acid, Sigma, St. Louis, MO), 0.12 M NaCl, 0.05 M KCl, 0.001 M $CaCl_2$, 0.005 M glucose, 1.5% (w/v) bovine serum albumin (Fraction V, batch WB 1370, Armour, Eastbourne, England), and 1.0 mg/ml of collagenase (Worthington, Freehold, NJ, batch CLS 46 N 026). Incubations were performed for 15 min at 37°C in a water bath where the flasks were shaken at a speed of 120 cycles/min. After 10 and 15 min, respectively, the flasks were vigorously mixed for 10 sec in a vibrating device (Super-mixer, Lab-Line Instruments, Melrose Park, IL).

After incubation the contents of the flasks were filtered through a nylon screen (250 μm pore size) to collect any remaining, nondisintegrated tissue. Thereafter the cell suspension was centrifuged at about 300 *g* for 3 min in a quick start and stop centrifuge. Fat cells and infranatant were now removed with a siliconized Pasteur pipette. The sediment was resuspended in 1.5 ml of the same HEPES-buffer with additions (except collagenase) as above, and the suspension was filtered through a 25-μm pore size nylon screen. This nylon screen was cut to fit into a 5-cm diameter Petri dish. After rinsing the filter with another 1 ml of buffer, the filter was placed upside down in the Petri dish containing 2.5 ml culture medium. Next the filter was rinsed, with the aid of mechanical scraping with a plastic spatula, with another 1.5 ml of medium. The nylon net was then removed and the cell suspension was distributed into a Multidish (Flow, Rockville, MD) for cell culture. Cells corresponding to at least one-fourth of the fat pad were used in each culture in a total of 1 ml of culture medium. The culture medium consisted of medium 199 with 5 mM glucose, 20% fresh human serum, 40 mU/ml of insulin (Vitrum, Stockholm, Sweden), and 0.1 mg/ml of sodium cefalotin (Keflin, Eli Lilly, Indianapolis, IN). The culture vessels were kept at 37°C in an incubator at 5% CO_2 in air with a water-saturated atmosphere. Medium was exchanged every second day.

Cell countings and biochemical analyses

Cells were removed from culture vessels by incubating them for 10 min at 37°C with collagenase (1.0 mg/ml) in buffer as described above. Cells were counted in a Fuchs-Rosenthal blood cell counting chamber with 0.2 mm depth and 1/16 mm^2 area.

Triglyceride synthesis from [U-^{14}C]glucose and glycerol release were determined as described previously (10). Lipoprotein lipase activity was assayed according to Nilsson-Ehle, Tornqvist, and Belfrage (11). Elution of activity from cells was performed for 5 min with a medium containing 5% (v/v) fasting serum and 1 IU/ml of heparin (Vitrum, Stockholm, Sweden) in medium 199. A second elution contained essentially no activity.

In experiments with binding of lipoprotein lipase, the first subculture of cells was used, and cells were divided into two new cultures. The subculture was allowed to grow until a confluent monolayer was obtained. Fresh medium was added containing about 5×10^5 adipocytes obtained from the epididymal fat pads of a fed Sprague-Dawley rat weighing about 300 g. These cells were liberated with collagenase as described above, but the incubation time was 30 min. The cells were washed twice with 5 ml of collagenase-free buffer before use.

Incubation times with the adipocytes are given in the Results section. After incubation the medium with adipocytes was removed, and the endothelial cells on the bottom were rinsed twice with 0.5 ml of medium without adipocytes. Elution of lipoprotein lipase activity was then performed with heparin as described above. Lipoprotein lipase activity was then determined in adipocytes, in adipocyte-containing medium, in medium used for washings, in heparin eluate, and finally in the heparin-eluted endothelial cells.

In one series of experiments, adipocytes were first incubated for 15 min in medium and then removed before adding only the medium to the endothelial cell cultures. The procedures described above were followed.

In order to control unspecific binding, culture vessels without endothelial cells were treated as above. This binding was corrected for by subtraction, and amounted to less than 20% of that bound with cells present.

Triglycerides were determined according to Carlson (12).

Microscopic procedures

Under the light microscope, cells were observed and photographed either after staining of the nuclei with crystal purple, or unstained with phase contrast optics.

For electron microscopy, cells were either removed

as described above, or fixed in situ on the bottom of the plastic Petri dishes (Flow, Rockville, MD). Both types of cell preparations were fixed with 1.25% glutaraldehyde (ultrastructural grade, Polaron, Watford, England) and 1% para-formaldehyde (Kebo, Stockholm, Sweden) in 0.15 M sodium cacodylate buffer (Merck, Darmstadt, West Germany) at pH 7.3 for 24 hr in the cold, rinsed in cacodylate buffer, and postfixed in 2% osmium tetroxide (Analytical Standards, Kungsbacka, Sweden). The specimens were dehydrated in a graded series of ethanol in water (50, 70, 90, 95, 99.5%, respectively), and embedded in Spurr's epoxy resin or Epon 812 (Taab Lab., Reading, England). Ultrathin sections were cut on an LKB type V Ultrotome, contrasted with uranyl acetate and lead citrate, and examined in a JEOL 100-CX electron microscope at an accelerating voltage of 80 kV.

For immunofluorescence studies of Factor VIII, cells were cultured in secondary cultures on glass coverslips. They were then rinsed twice in phosphate-buffered saline (PBS) (pH 7.2) and fixed for 10 min in acetone at 0°C, followed by another rinse in PBS. The preparations were then incubated for 30 min at 37°C in a moist chamber with either rabbit anti-human F VIII (AO82, DAKO-immunoglobulins Ltd, Copenhagen, Denmark) or, as control, rabbit anti-guinea pig IgG (CZ 108, DAKO) (both antisera diluted 1:10 in 4% bovine serum albumin in PBS). After three rinses in PBS for 15 min, incubations were performed with fluorescin-conjugated anti-rabbit IgG from swine (F2190, DAKO) in a moist chamber for 30 min at 37°C (diluted 1:10 in 4% bovine serum albumin in PBS). After three more rinses in PBS (pH 8.0) for 15 min, the preparations were mounted in buffered glycerin and a differential count of fluorescent cells was performed in a fluorescence microscope.

RESULTS

The aggregated cells collected on the 25-μm filter are seen in **Fig. 1**. Typically segments of capillaries were found among groups of cells and single cells.

It was not possible to break the cell aggregates with low ionic strength, hyaluronidase, elastase, heparin, or lowering of pH. However trypsin not only broke up the cell aggregates but also destroyed a major part of the cells. DNA:ase had no effect, demonstrating the absence of nonspecific binding due to cell breakage (not shown).

Cell culture

The cells tended at first to stick to the bottom mainly as aggregates, and from these new cells began to appear, spreading radially until a monolayer was formed on the bottom after about 4–6 days. In primary cultures cells

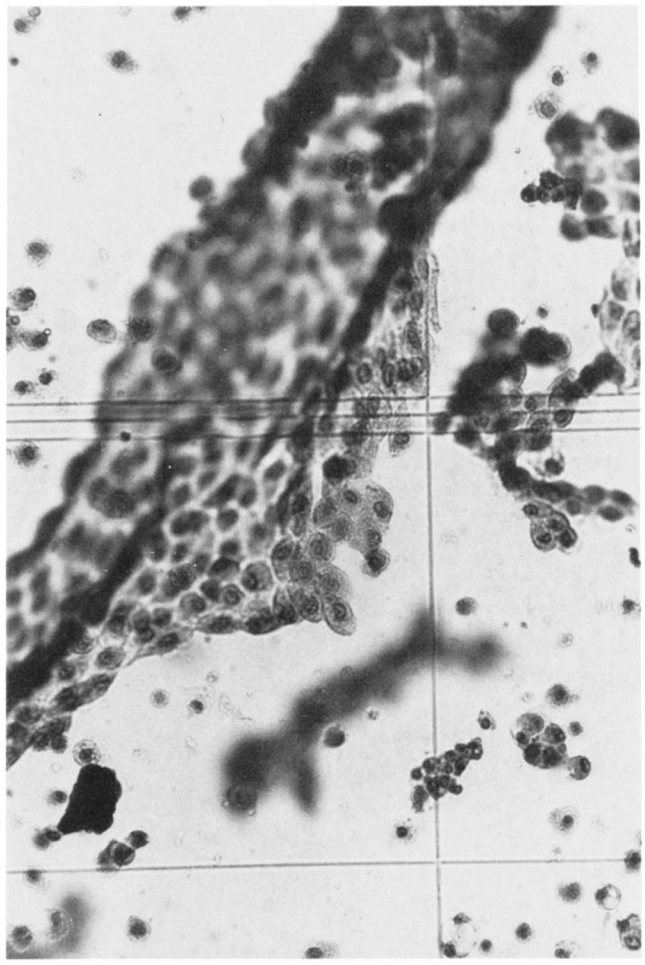

Fig. 1. Cells recovered on a 25-μm filter before culture. Staining with crystal purple. Average cell diameter 12–15 μm.

forming adipocytes were often seen (<10% of total cells) (**Fig. 2**). Subculturing (1:1 or 1:2) made these fat cells disappear. Further passages of the cells were possible, but after another one or two passages the appearance of cells with other morphologic characteristics as well as a poor rate of replication were observed.

The cells in the first subculture had a fairly large, lucid cytoplasm and formed a typical, confluent monolayer resembling a cobblestone pavement (**Fig. 3** and **Fig. 4**). Electron microscopy of these cells revealed a large number of micropinocytotic vesicles, together with a fairly well-developed rough endoplasmic reticulum, mitochondria, and Golgi apparatus (**Fig. 5**). The cells contained large numbers of intermediate (100 Å diameter) filaments and smaller amounts of actin (60 to 80 Å diameter) filaments (Figs. 5 and 6). Neighboring cells formed junctional complexes with both tight and gap junctions (**Fig. 6**). These complexes persisted even if the cells were treated with collagenase as described above.

In differential counts more than 95% of the cells

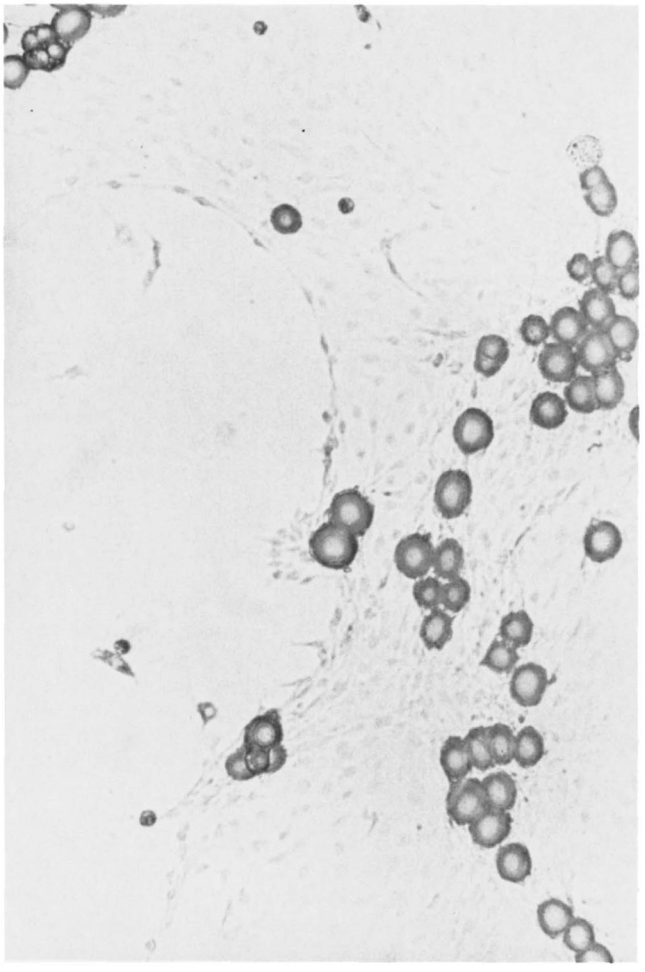

Fig. 2. Primary culture of cells recovered on a 25-μm filter. Phase contrast, no staining. Average fat cell diameter about 25 μm.

showed fluorescence in these secondary cultures after exposure to Factor VIII antisera; while in controls, exposed to IgG antisera, no fluorescence was seen.

Functional characteristics

Table 1 shows the very low rate of synthesis of triglycerides in these cells, whether obtained directly from the isolation procedures or from culture, in comparison with the adipocytes from the same epididymal fat pad. Triglyceride hydrolysis was also very low and was not stimulated by norepinephrine (**Table 2**). After long-time culture (several weeks), no lipid granulation was visible even if a triglyceride emulsion was added to the cell culture, a medium in which precursor cells to adipocytes collect lipid very efficiently (10).

Lipoprotein lipase was present in the cells obtained directly from the isolation procedure (**Table 3**). There was, however, a large, nonsystematic variation in these results. In the primary cultures lipoprotein lipase was also present, and this activity could be removed totally by heparin elution. After heparin elution these cells did not form any new lipase activity upon further incubation, while this was the case with adipocytes incubated in parallel (Table 3).

It was suspected that the presence of a few preadipocytes forming adipocytes in the primary culture might produce the lipase activity released from the endothelial cells, because such cells are able to produce a large amount of lipoprotein lipase during their development (13). Therefore, endothelial cells from the first subculture, free of preadipocytes, were analysed for lipoprotein lipase activity and were found to lack such activity (Table 3).

Binding of lipoprotein lipase

The results referred to above suggested that the endothelial cells were able to bind lipoprotein lipase. Therefore, at first, adipocytes were incubated in the fortified medium 199 together with endothelial cells in subculture. The endothelial cells, originally free from

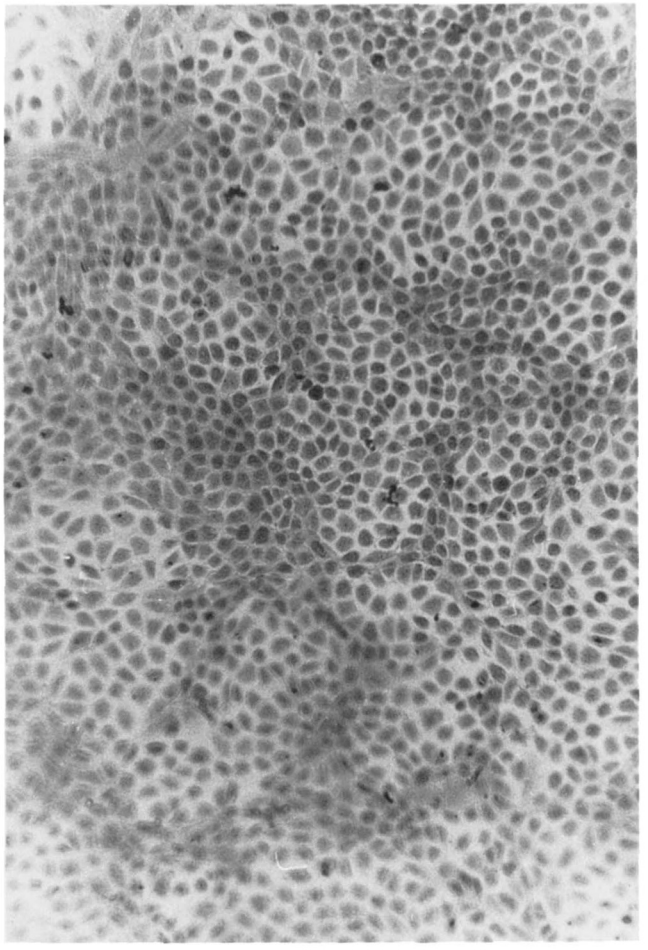

Fig. 3. First subculture of cells recovered on a 25-μm filter. Staining with crystal purple. Average cell diameter 12–15 μm.

lipoprotein lipase activity, then had picked up such activity that was possible to elute with heparin (**Table 4**).

Fig. 7 shows similar results in more detail. Adipocytes added to the medium contained lipoprotein lipase that was already detectable in the medium after 15 min and then increased to a steady level. After 4 hr no activity could any longer be eluted from the adipocytes. Although absent from the outset (not shown), the endothelial cells had picked up lipoprotein lipase activity at 15 min, apparently already at a maximal level that remained constant throughout the experiment. This activity was eluted totally by heparin.

In an attempt to follow the initial binding of lipase activity, further experiments were performed (**Fig. 8**). In order to expose the endothelial cells to enough lipase activity from the beginning, adipocytes were first incubated in medium (without heparin) into which lipoprotein lipase activity was then released. When adding this medium without the adipocytes to subcultures of endothelial cells, activity was rapidly bound to these cells, and was apparently maximal within 30 min. Concomitantly, activity disappeared from the medium.

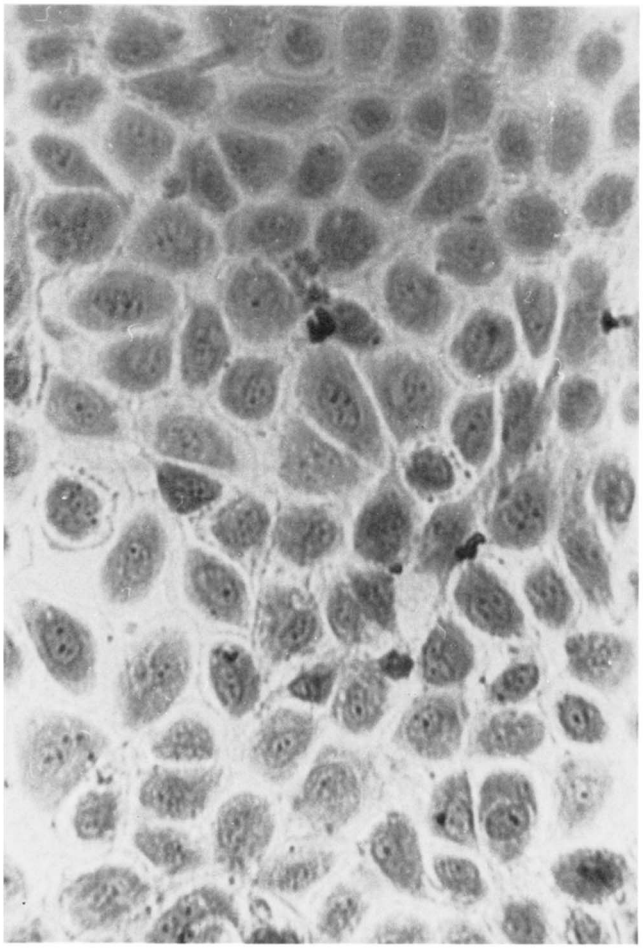

Fig. 4. First subculture of cells recovered on a 25-μm filter. Staining with crystal purple. Average cell diameter 12–15 μm. Same cells as in Fig. 3 at higher magnification.

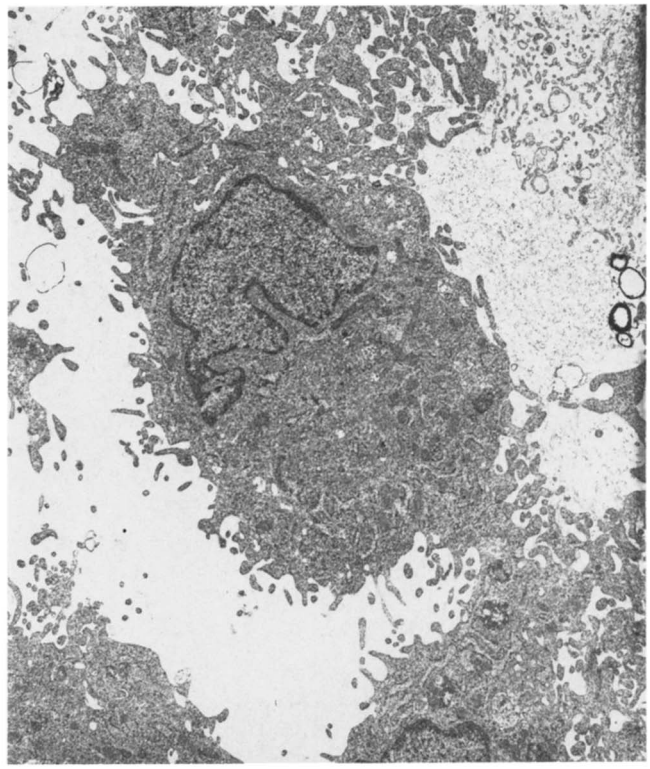

Fig. 5. Electron micrograph of cell in first subculture, fixed after removal from Petri dish with collagenase. Many micropinocytotic vesicles, mitochondria, and e.r. profiles are observed. Magnification 7,300×.

DISCUSSION

The cells obtained with the procedures described here have the typical light and electron microscopic characteristics of capillary endothelial cells, including the growth pattern in vitro with the formation of a confluent monolayer and morphology as seen both in the light and the electron microscope. Among ultrastructural details, the appearance of intercellular junctional complexes and micropinocytotic vesicles are particularly important. The predominance of intermediate filaments as compared to thin filaments is also characteristic for endothelium (14). The lack of Weibel-Palade bodies suggests that the cells are derived from capillary endothelium, since this is known to contain very few such organelles. Finally, the observation of a well-developed endoplasmic reticulum and Golgi apparatus is in agreement with studies on early passages of endothelial cells from other laboratories (6). A majority of the cells showed the presence of Factor VIII. In conclusion,

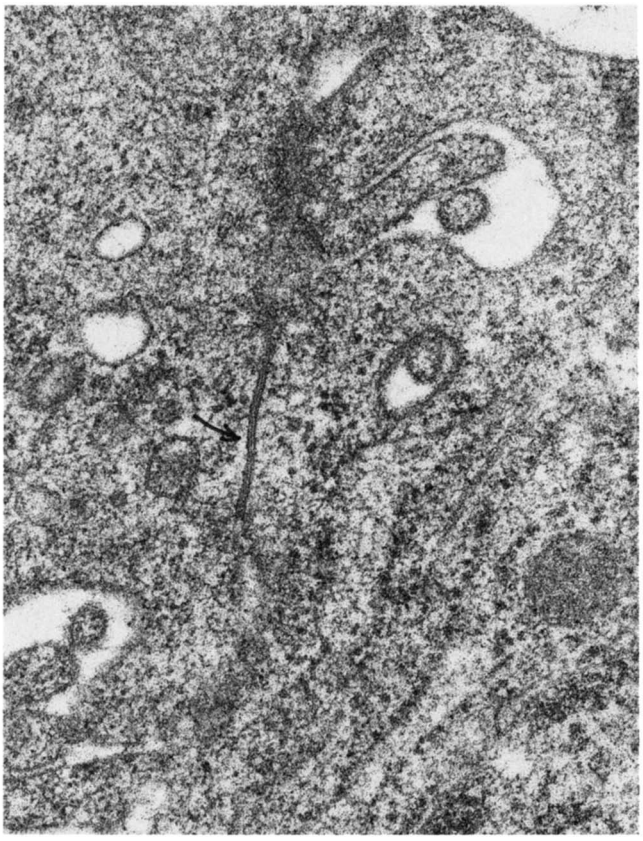

Fig. 6. Area of contact between neighboring cells in first subculture. A tight junction is formed between the cells (arrow). Both cells are rich in intermediate filaments. Electron micrograph. Magnification 69,000×.

these observations characterize the cells as endothelial cells from the microvessels of adipose tissue.

The procedure used to obtain these cells is based on removing tissue remnants on a large-mesh net and then, after adipocyte removal, collecting the capillaries or parts of capillaries on a fine-mesh net, which allows single cells to pass. Exposure of the cells to collagenase for as short time as possible is essential for cell survival. A minimum of cells has to be incubated; fewer cells than those obtained from one-fourth of an epididymal fat pad did not grow. Preparations from larger rats often showed contamination of fibroblasts in cultures. With the exception of triglyceride emulsion, the culture medium with its additions is identical to that used for culture of adipocyte precursor (10). The necessity of glucose and insulin has not been systematically tested. This procedure allowed adipocyte precursors in the primary culture to fill up with lipid and simplified their removal by flotation during subculture procedures. Using volumes of human serum other than 20%, or using fetal calf serum, apparently does not improve the results. Adhering strictly to these procedures gives homogenous, growing cultures in at least two experiments out of three. It has not been possible to trace the reason(s) for lack of success in the remaining cases.

The functional characteristics examined here have been those typical of adipose tissue and adipocyte functions, viz. deposition and release of triglycerides. These results demonstrate clearly the lack of relationships between adipocytes or their precursor cells and the endothelial cells. The adipocyte series of cells have functions for triglyceride synthesis and release, the latter possible to stimulate with catecholamines, as well as a marked synthesis of lipoprotein lipase (10), all in contradistinction to the endothelial cells. Prolonged culture in triglyceride-rich media, allowing adipocyte precursors to accumulate lipid rapidly, did not produce any evidence for triglyceride accumulation in the endothelial cells in spite of the fact that lipoprotein lipase had been shown to be present in this cell population. This

TABLE 1. Triglyceride synthesis of endothelial cells and adipocytes from the epididymal fat pad[a]

Adipocytes	1.0 ± 0.1
Cells collected on a 25-μm filter	0.04 ± 0.01
Endothelial cells in primary culture (10 days)	0.11 ± 0.02

[a] Results are given as μmol of [U-^{14}C]glucose incorporated into triglyceride per 10^8 cells per hr. For further details, see Experimental Procedures. Means ± SEM. n = 3–6. Adipocyte diameter, 33 ± 5 μm.

TABLE 2. Glycerol release from endothelial cells and adipocytes from the epididymal fat pad[a]

	Basal	Norepinephrine
Adipocytes	15 ± 6	164 ± 14
Cells collected on a 25-μm filter	4 ± 1	5 ± 1
Endothelial cells in primary culture (10 days)	2 ± 1	3 ± 1

[a] Results are given as μmol of glycerol released per 10^8 cells per hr. Means ± SEM. n = 3–5. Adipocyte diameter, 33 ± 5 μm. Norepinephrine was present in a concentration of 5×10^{-6} M.

TABLE 3. Lipoprotein lipase activity in endothelial cells and adipocytes from the epididymal fat pad[a]

Adipocytes	31 ± 2
Heparin eluate from d:o	25 ± 3
Eluted adipocytes after another 30′ of incubation	20 ± 2
Cells collected on a 25-μm filter	11 ± 16
Endothelial cells in primary culture (10 days)	12 ± 3
Heparin eluate from above	14 ± 2
Eluted cells	0 ± 0
Eluted endothelial cells after another 30′ of incubation	0 ± 0
Endothelial cells in first subculture	0 ± 0

[a] Results are given as mU per 10^8 cells. Means ± SEM. n = 3–6. Adipocyte diameter, 31 ± 7 μm. For further experimental details, see Experimental Procedures.

TABLE 4. Binding of lipoprotein lipase activity to endothelial cells incubated in the presence of adipocytes[a]

Adipocytes	45[b]
Endothelial cells before incubation with adipocytes	0
Adipocytes after incubation	14
Endothelial cells after incubation with adipocytes for 1 hr	120
Above after heparin elution	0

[a] To endothelial cells in first subculture after formation of confluent monolayer were added 6×10^5 adipocytes isolated from the epididymal fat pad of a 310-g fed Sprague-Dawley rat. For further experimental details see Experimental Procedures.

[b] Results given as mU per 10^8 cells. Means of duplicates in one representative experiment.

evidence demonstrates the functional difference between the endothelial cells and cells in the adipocyte series after determination, and demonstrates the inability of endothelial cells to be converted into cells of the adipocyte series. This does not exclude, of course, the possibility that adipocytes and endothelial cells of adipose tissue might have a common stem cell, with endothelial cells and adipocytes as end products.

The culture of endothelial cells from adipose tissue capillaries has previously been described by Wagner and Matthews (9). The source of these cells was intact capillaries from fairly large rats. The cultures contained cells other than endothelial cells, which had to be removed by repeated treatments with thimerosal. The present procedure seems to have advantages in the ease of preparing cells for culture, the homogeneity of the preparation without treatments with enzyme inhibitors, particularly in subculture, and an apparently faster rate

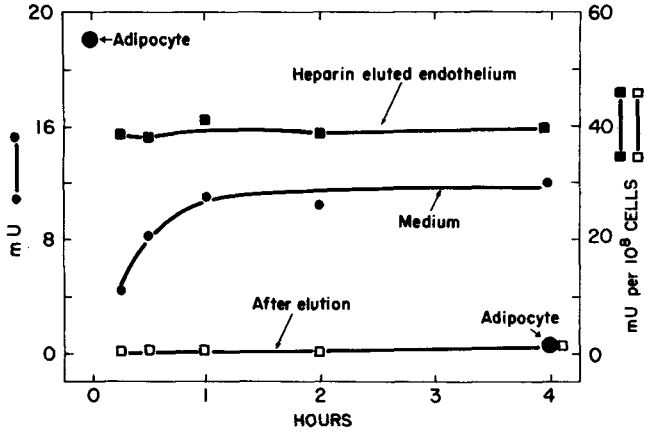

Fig. 7. Binding of lipoprotein lipase from adipocytes by endothelial cells. Adipocytes ($5 \cdot 10^5$) added to each of five first subcultures of endothelial cells. Lipoprotein lipase activity in adipocytes, elutable with heparin before and after 4 hr incubation (large filled circles). Lipoprotein lipase in medium (small filled circles), and eluted from endothelial cells (filled squares), or after elution of endothelial cells (open squares) measured at indicated times. Activity expressed per cell basis in adipocytes and endothelial cells, and in the total medium. No measurable activity in endothelial cells before incubation with adipocytes. One representative experiment out of three performed.

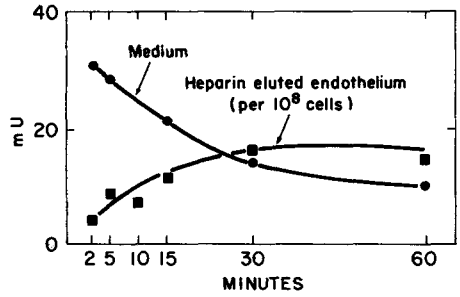

Fig. 8. Binding of lipoprotein lipase from adipocytes by endothelial cells. Activity first eluted into medium (without heparin) from about 10^6 adipocytes, and medium without adipocytes then added to six first subcultures of endothelial cells. Symbols and expression of activity as in Fig. 7. No measurable activity in endothelial cells before incubation with lipoprotein lipase-containing medium. One experiment out of two performed.

of replication, which might have to do with the younger age of the donor animals.

Evidence has been given for binding of lipoprotein lipase activity from adipocytes to the endothelial cells. The bound lipase could be released by heparin. Activity then did not reappear in the absence of lipase in the incubation medium, showing that these cells probably do not synthesize lipoprotein lipase, as is known to be the case for adipocytes (15). Knowing this, it is likely that the activity found in endothelial cells before culture had been picked up from fat cells or their precursors, present to some extent in the primary cultures, and during the preparation procedures of the intact fat pad.

The binding experiments furthermore showed that the binding is a rapid process. When the enzyme was bound to the endothelial cells, its inactivation seemed to be prevented (Fig. 8). The enzyme activity bound was comparable to that found in adipocytes. It was removable by heparin. Further details of binding kinetics and other characteristics will have to await other studies with purified enzyme.

The present concept of the physiological integration of the triglyceride uptake process in adipose tissue rests on the hypothesis that lipoprotein lipase is synthesized in adipocytes and bound to the endothelial cells of the adipose tissue capillaries where triglyceride hydrolysis takes place (16, 17). This hypothesis is built mainly on the finding of a momentary release of lipoprotein lipase by heparin perfused through the tissue (18), the interactions between lipoprotein lipase and glucose aminoglucans (19), and the fact that intact triglycerides cannot be taken up in adipocytes to any significant extent (20). The present work supports this hypothesis because it has been shown that lipoprotein lipase can be bound to endothelial cells from adipose tissue capillaries, and this binding can be released by a glucose aminoglucan (heparin), which is also the case in the intact, integrated system (18). Furthermore, the lipase is not synthesized

by the cells but can be transferred in vitro from adipocytes. The system of homogenous endothelial cells in culture from adipose tissue capillaries described here should be useful in further reconstruction experiments to test the hypothesis mentioned.

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