Quantitation of different cells in the epididymal fat pad of the rat

Per Björntorp, Majvor Karlsson, Lars Gustafsson, Ulf Smith, Lars Sjöström, Massimo Cigolini,[1] Gunnar Storck, and Per Pettersson

Clinical Metabolic Laboratory of Department of Medicine I, Sahlgren's Hospital, University of Gothenburg, Sweden

Abstract   To determine the number of adipocytes and cells developing into adipocytes (preadipocytes) in the epididymal fat pad of normal Sprague-Dawley rats, two methods were developed. Liberation of all cells from the tissue was obtained by a combination of lytic enzymes and mechanical treatment with only a limited loss of cell integrity; with large tissue masses, an initial perfusion was necessary. These cells were cultured in medium 199 supplemented with serum, glucose, insulin, a triglyceride emulsion, and methyl cellulose to form a culture medium with high viscosity in which it has been shown that the cells do not multiply. In this medium some of the cells developed into adipocytes and could be recognized and counted. The results show that there are about twice as many preadipocytes as mature adipocytes in the smallest rats examined (about 50 g). With increasing weight and age the mature adipocytes increased while the number of preadipocytes seemed to be constant up to a weight of about 150 g, after which they continuously diminished and could not be found in rats weighing more than 300 g. Here the number of mature fat cells had reached a constant level. These results are consistent with the formation of new preadipocytes up to a rat weight of about 150 g. In normal rats these cells successively fill up with triglyceride and disappear at a body weight of about 300 g when they have been transformed into mature adipocytes. The results are also consistent with the concept that no new adipocytes are formed spontaneously in the adult Sprague-Dawley rat. It was shown, however, that in media without methyl cellulose, isobutylmethylxanthine probably could induce the formation of new adipocytes in the cells isolated from all rats, including the heaviest (oldest). This finding shows that the potential of cells to develop into adipocytes also seems to exist in the adult rat under certain circumstances.

Supplementary key words adipocytes · preadipocytes · tissue culture

Information on the morphology of adipose tissue has proved valuable in research on how adipose tissue develops as well as in clinical situations (1, 2); However, definite conclusions are not possible with currently available methods for sizing and counting fat cells, because these methods (3–5) exclude fat cells that do not contain a certain minimal amount of triglyceride. The presence of such cells has been established by the use of methods that label fat cell precursors in vivo and harvest them after their development into mature adipocytes (6). However, estimation of the number of precursor cells in relation to that of the mature fat cells has never been made. This information is necessary to allow an evaluation of presently available data on adipose tissue development.

In a recent report (7) it was disclosed that, in preparations of epididymal fat pads of small rats, cells are present that can multiply and develop into cells identical in morphology and function with mature adipocytes. The method used in that study does not allow the quantitation of the fat cell precursors, however. In order to achieve this goal methodological developments are necessary. First, a method is needed whereby the fat cell precursors can be recognized and counted. Since no certain morphological characteristics are known for these cells, an approach to this problem was selected in which the cells were first isolated and then allowed to develop in culture into mature fat cells in order to circumvent the difficulties in characterizing the preadipocytes. To allow quantitation, the cells had to be isolated quantitatively from the tissue and multiplication of these cells had to be avoided. The present report describes these methodological developments as well as the results obtained in studies with adipose tissue from rats of different ages.

EXPERIMENTAL PROCEDURES

Preparation of tissues

Male Sprague-Dawley rats of different weights and ages (see Results) were used. They were acclimatized

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[1] Research fellow from the Medical Clinic III, University of Padua, seat in Verona, Italy.

in the laboratory for at least 3 days and given ad libitum tap water and commercial rat pellets (EWOS, Södertälje, Sweden) containing 5, 55, and 22.5% fat, carbohydrate, and protein, respectively, plus minerals and vitamins. 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 total epididymal fat pad was removed from the epididymis and the vessels were cut off at the point where they enter the fat tissue. The vessels were then opened with thin scissors as far as possible in the pad and blood was rinsed off. Next the tissue was cut into pieces of approximately 25 mg and incubated to liberate the cells.

Liberation of fat cells

In order to obtain as complete liberation of the cells as possible, procedures were tested to improve the ordinary collagenase incubation method by the addition of other enzymes and by application of mechanical procedures. The following method proved to be optimal.

Incubations were performed in 50-ml siliconized Erlenmeyer flasks containing 10 ml of a solution, pH 7.4, with final concentrations of 0.1 M HEPES buffer (hydroxyethylpiperazine-ethanesulfonic acid, Sigma, St. Louis, MO) 0.12 M NaCl, 0.05 M KCl, 0.001 M $CaCl_2$, 0.005 M glucose, and 1.5% (w/v) bovine serum albumin (Fraction V, batch WB 1370, Armour, Eastbourne, England). To this flask 1.5 mg/ml of collagenase (Worthington, Freehold, NJ, batch CLS 46N026), 0.3 mg/ml of elastase (Sigma, batch 74C-8330), and 0.3 mg/ml of hyaluronidase (Sigma, Type I, batch 33C-2240) were added. Trypsin (Sigma, Type I, batch 112C-8230) had no additional effect and produced cell destruction. The pad was dissected into 25-mg pieces and then incubated for 30 min at 37°C in a water bath where the flasks were shaken at a speed of 120 cycles/min. After 15 and 30 min, respectively, the flasks were vigorously mixed for 10 sec in a vibrating device used for mixing the contents of test tubes (Super-mixer, Lab-Line Instruments, Melrose Park, IL).

After a 30-min incubation the contents of the flasks were filtered through a nylon screen (250 μm pore size) to collect the remaining, nondisintegrated tissue. The fat cells were now allowed to float to the surface after which the infranatant medium was removed by aspiration. They were then washed twice with 3 ml of incubation medium and these washings were pooled with the infranatant medium.

The tissue residues were subjected to further treat-ment. They were incubated again with 5 ml of fresh incubation solution as described above, and then aspirated twice through a siliconized syringe with 2-mm i.d.; this was repeated with another siliconized needle of 1.2 mm i.d. The incubation was continued for another 30 min with shaking and mixing as above. After 30 min the contents were again filtered through a 250-μm filter to recover the tissue residues. This material was collected. The cells of the filtrate as well as those of the previous infranatant with its washings were pelleted separately by centrifuging at 400 g for 4 min for further processing.

Perfusion of the epididymal fat pad

To facilitate disintegration of the large fat pads of the oldest rats, perfusion of the right epididymal fat pad was performed through the internal spermatic artery. The rat was anesthetized and a plastic cannula was inserted into the abdominal aorta and held in place at the level below the internal spermatic artery by a ligature around the aorta. A ligature was also placed above the renal arteries. These and all other arteries connected to the aorta between the two ligatures were then carefully ligated, except the right internal spermatic artery. A perfusion pump was now started at a speed of 0.33 ml/min. The perfusion fluid was HEPES buffer with albumin, glucose, salts, and enzymes as described above. The perfusion was performed for 10 min. The tissue was then removed in one piece and placed in 10 ml of perfusion medium where it was minced into 25-mg pieces. This was followed by a conventional incubation in the perfusion medium for another 60 min including shaking, mixing, and aspirations through injection needles as described above for unperfused tissues.

Cell culture

Cell cultures were performed in Leighton tubes at 37°C in medium 199 with 5 mM glucose supplemented with 20% (v/v) fresh human serum, 40 mU/ml of insulin (Vitrum, Stockholm, Sweden), 0.5% (w/v) of a triglyceride emulsion in lecithin (Intralipid, Vitrum, Stockholm, Sweden), and 0.1 mg/ml of sodium cefalotin (Keflin, Eli Lilly, Indianapolis, IN). Other supplementations were as stated in the text. Isobutyl-methylxanthine was obtained from Aldrich, Milwaukee, WI. These culture media were changed at least every second day. Some of the cultures were performed in a medium containing 1.3% (w/v) methyl cellulose (Methocel, Dow Chemical Co., Midland, MI). In these cultures the cells obtained after incubations in enzyme solutions as described above were suspended directly in the viscous medium. The number of cells per tube was about 10^5. Rat skin fibroblasts

were obtained by cutting small pieces of tail skin. Cover glasses kept these pieces attached to the bottom of culture tubes and, when cultured in medium 199 with supplementations as above, cells grew out from the edges of the tissue pieces.

Cell counting and other measurements

Cells for counting were removed from the culture tubes without methyl cellulose by incubating them for 10 min at 37°C with 0.125% (w/v) trypsin (Sigma, Type I, St. Louis, MO) in 0.15 M phosphate buffer, pH 7.0. Cells were counted in a Fuchs-Rosenthal blood cell counting chamber with 0.2-mm depth and 1/16 mm² area.

In cultures with methyl cellulose, cells were counted by one of the following methods. Direct counting of cells could be performed by dividing the bottom area of the culture tube into a number of equally sized fields and counting cells all through the Methocel medium in a number of fields selected at random. Monovacuolar cells with a diameter >20 μm were counted as adipocytes. A simpler procedure, which proved to give similar results, was to perform a differential count by determining the relation between adipocytes and other cells among a total of about 400 cells examined in different parts of the tube. The number of cells cultured was known and the total number of fat cells and other cells could then be calculated. This method requires that no cells disappear or are added during culture. The latter error is excluded by the demonstration that no significant cell multiplication occurred in the Methocel medium (see below). The similarity in results with the direct method suggests that no cells disappeared during culture.

Fat cell diameter in the original, intact adipose tissue was determined on frozen slices as described previously (4). The diameter of cultured cells was measured by an ocular eyepiece. Incubations for determinations of DNA synthesis from [methyl-³H]-thymidine (NET-027Z, batch 824-204, New England Nuclear Co, Dreieichenhain, West Germany) were performed for 1 hr in the same culture media as above but containing 1 μCi of labeled thymidine and 50 μmol of nonlabeled thymidine (Sigma, grade, batch 63C-0790). DNA was then isolated (see below), dissolved in 1N ammonium hydroxide, and counted in a liquid scintillation counter. DNA was determined according to the method of Kissane and Robins (8) after delipidization in acetone and diethylether as in the method of Nilsson-Ehle, Tornqvist, and Belfrage (9). Protein determinations were performed according to Lowry et al. (10). Triglyceride analysis was performed according to Carlson (11). Gradient centrifugation of cells in density fractions <1.040,

1.040–1.055, and 1.055–1.070 g/ml was performed as described previously (7).

RESULTS

Liberation of cells from the epididymal fat pad

Table 1 shows that with collagenase in the incubation medium the cells of the epididymal fat pads of small rats could be liberated to an extent where no residue remained. With larger rats, however, 37% of the total protein was obtained in the residue. This seemed to improve to 23% after passing the sediment through needles.

With addition of elastase and hyaluronidase there was again a complete liberation of protein from the tissue of small rats and no residue. The first 30 min of incubation seemed to yield almost all protein not present in fat cells, because subsequent incubation after treatment with needles yielded only an additional 9%. With large rats addition of elastase and hyaluronidase gave some more protein in the sediment after passing the tissue remnants through the needles, but there was still protein remaining in the final residue. Recovery was essentially complete.

For this reason perfusion of the tissue was performed with the enzyme solution before mincing and incubation. **Table 2** shows that this procedure gave no residue, indicating a complete liberation of protein from the fat pads of the larger rats, also. DNA measurements in the fractions gave principally the same re-

TABLE 1. Liberation of protein (%) from the epididymal fat pad of rats by incubating minced tissue in enzyme solutions

	Collagenase		Collagenase + Elastase + Hyaluronidase[f]	
	Large Rats[a]	Small Rats[b]	Large Rats	Small Rats
Fat cells	32 (35)[e]	35	38	36
First sediment[c]	30 (28)	45	25	55
Second sediment[d]	15 (—)	20	27	9
Residue	23 (37)	0	13	0

Results given as % of total sedimentable protein at 400 g for 4 min plus protein in fat cells. One representative experiment out of 3–6.

[a] Large rats: 220–340 g.

[b] Small rats: 45–100 g.

[c] First sediment: sedimentable protein after first 30 min of incubation.

[d] Second sediment: sedimentable protein after another 30 min of incubation of the residue obtained after first 30 min. Residue passed through needles as described in Experimental Procedures.

[e] Results of first column within parentheses: passing through needles not performed.

[f] Recovery of DNA: 84–91% (n = 3; small rats).

TABLE 2. Liberation of protein and DNA (%) from the epididymal fat pad of rats (330–420 g) by perfusion with and incubation of minced tissue in enzyme solutions[a]

	Not Perfused (protein)	Perfused		Trypan Blue[d]
		(protein)	(DNA)	
Fat cells	39	36	23	
First sediment[b]	29	40	59	10
Second sediment[c]	20	23	18	14
Residue	12	1	0	13

[a] Results given as % of total sedimentable protein or DNA at 400 g for 4 min plus protein or DNA in fat cells. One representative experiment out of 3–5.

[b,c] See Legends c and d, respectively, of Table 1.

[d] Results given as % cells not excluding stain.

sults, the somewhat higher percentage of protein than of DNA in the fat cell fraction probably being explainable by the protein content of the washing solution. This washing solution consisted of the incubation medium with albumin and enzymes and remained in the fat cell fraction in the protein measurements. The trypan blue exclusion test showed 14% or less of cells not excluding the stain.

Culture of cells in medium containing methyl cellulose

When cells obtained from the epididymal fat pad of small rats were cultured in the supplemented medium 199 containing methyl cellulose, they remained suspended in the viscous medium and did not assume the typical fusiform shape seen in medium 199 without methyl cellulose. There was no certain microscopic evidence for a multiplication of the cells. They remained as single or multiple units in the medium in repeated photographs of the same regions of the cultures over several weeks.

Results of incubations with labeled thymidine and subsequent isolation of DNA are shown in **Table 3** for cells from 40–45 g rats cultured with or without Methocel for 7 days. The cells in Methocel incorporated insignificant amounts of thymidine label into DNA while the control flask without Methocel showed a marked synthesis of DNA.

TABLE 3. The incorporation of [methyl-³H]thymidine in DNA in cells cultured with and without methyl cellulose in the medium

Culture Conditions (7 days)	Cpm per Culture Tube	Cpm per ng DNA
Without methyl cellulose	3251	10.2
With methyl cellulose	234	0.4

Incubations of equal number of cells in six culture tubes with or without methyl cellulose in fortified medium 199 with thymidine (see Experimental Procedures). Mean of triplicate determinations in one representative experiment.

After some days, accumulation of a structure-free material was seen in some of the cells. This continued until cells of the size of 30–40 μm, dominated by one vacuole of this material, were seen after about 3–4 weeks together with cells free of this material (**Fig. 1**).

At this stage the Methocel medium could be diluted with medium 199 and the monovacuolar cells collected by flotation (**Fig. 2**). Oil red O staining and triglyceride analysis revealed the triglyceride nature of the contents of the cells, which were microscopically identical with mature fat cells. At this stage the cells could also be quantitated by counting or, simpler, by differential counting in the Methocel medium if an appropriate concentration of cells per medium volume was cultured. In this case cells at different depths of the Methocel medium had to be brought into focus and counted.

Number of cells in the epididymal fat pad

Rats of different ages and weights were next examined for the cellular contents of the epididymal fat pads. **Fig. 3** shows that with increasing animal weight the epididymal fat pad weight also increased. The total number of cells apparently also increased in a linear fashion with rat weights in this range. The total number of mature adipocytes, however, seemed to reach a maximum in rats weighing about 200 g and apparently did not change at higher weights. The number of preadipocytes was fairly abundant at rat weights up to 150–200 g. In the smallest rats examined the number of preadipocytes was about twice the number of adipocytes. The preadipocytes then declined both in relative numbers in relation to the adipocytes and in absolute numbers until they were no longer detectable at rat weights of about 400 grams. Preadipocytes were not obtained in control experiments where the fat pad cells of the largest rats were liberated by perfusion plus incubation (not shown).

Culture of cells in medium without methyl cellulose

The same preparations of cells as used in the experiments shown in Fig. 3 were also cultured in fortified medium 199 without Methocel. These results are shown in **Fig. 4.** As described before (7) the cells obtained in the density fraction of <1.040 g/ml contain at least 30% (w/v) of triglyceride. These cells are adipocytes or develop quantitatively into adipocytes, at least in small rats (7), while the cells in the fraction 1.055–1.070 g/ml contain very little or no triglyceride. Fig. 4 then shows that there seemed to be some spontaneous development of cells that multiplied and filled up with triglyceride to form adipocytes in the smallest rats as described before (7). With increasing rat weight this tendency decreased and, in the largest
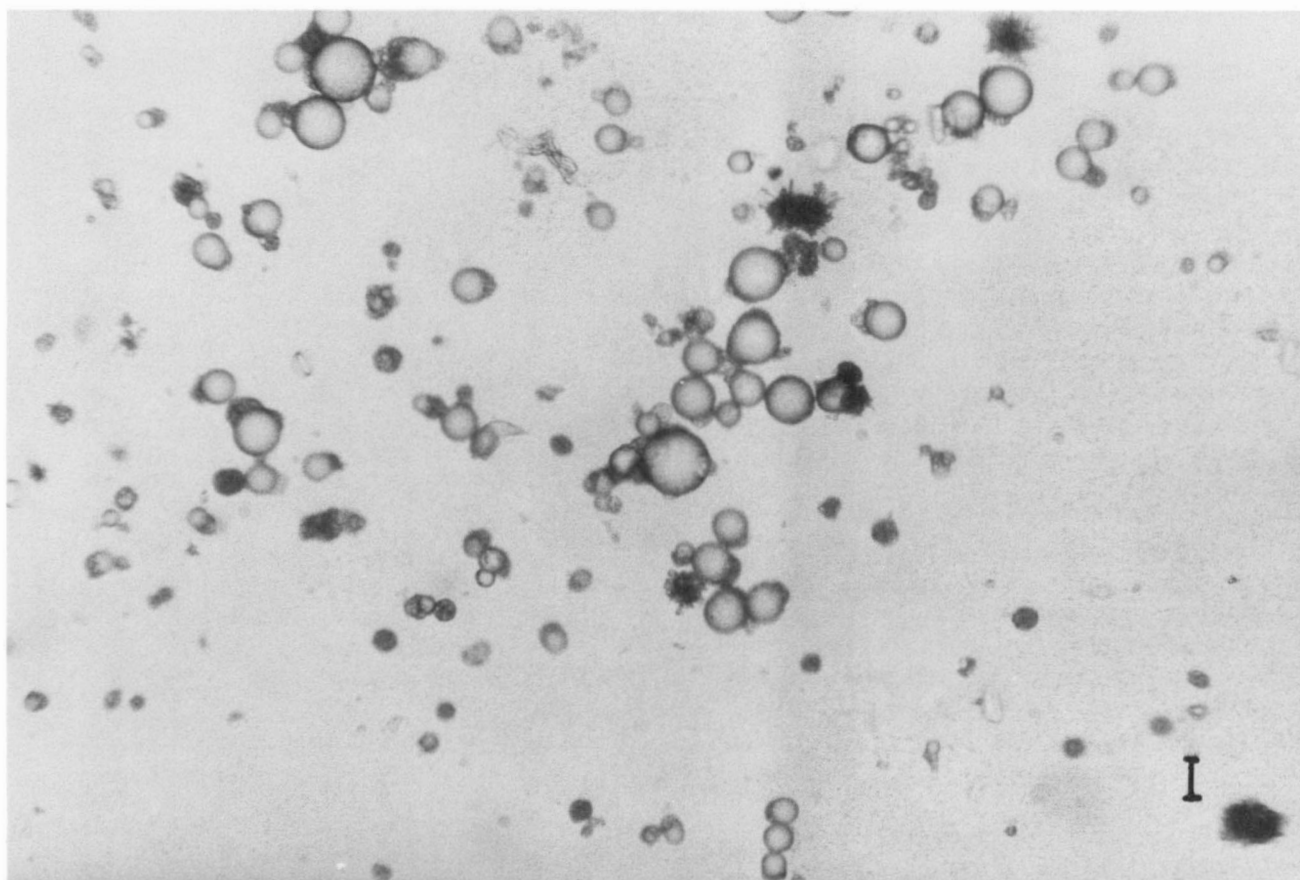
Fig. 1. Cells from a 54-g rat in culture for 3 weeks in medium containing methyl cellulose. Insert: 50 μm. To obtain a sufficient number of cells in one picture the number of cells cultured in this tube was about 10-fold higher than when cells were cultured for quantitation purposes.

rats, no spontaneous development was seen during this period. However, with 0.5 mM isobutylmethylxanthine there was a clear shift in the density profiles of the cells, which now were dominated by the lightest fraction at all rat weights.

Fibroblasts from rat tail skin that were cultured under similar conditions with or without Methocel and with or without isobutylmethylxanthine showed only minor lipid granules even after 6 weeks of culture (**Fig. 5**). Fat cell ghosts obtained by exposing fat cells to hypotonic solutions and subsequent recovery by centrifugation did not multiply or grow in any of the systems.

DISCUSSION

Liberation of cells from the epididymal fat pad

Different means were tried to obtain a total liberation of the cells in the epididymal fat pad while preserving cellular integrity; e.g., treatment of the tissue with enzymes with lytic effects on the tissue stroma combined with mechanical treatment. With tissues from small rats, collagenase alone sufficed while with tissues from larger animals, additional enzymes had to be added in optimal concentrations. This gave a yield of more cells but, judging from the protein determinations, the liberation of cells from the tissue residue was still incomplete. To obtain this the enzyme solution had to be perfused through the tissue. Cells so treated were damaged only to a small extent as judged from the trypan blue exclusion test. Furthermore, there was no evidence of extensive damage of the fat cells as judged from the virtual absence of free floating fat. Additionally, density gradient centrifugations did not reveal any fat cell ghosts at the expected density of ghosts (7) in these preparations. This is probably due to the fact that the adipocytes were removed at an early stage of incubation, before mechanical treatment. Perfusion of adipose tissue was previously performed by Rosell (12) in the dog and by Robert and Scow (13) in the rat. It proved important to use a low perfusion rate in the present procedure in order to fill up the vessels with the perfusion fluid. Since this was the only aim of the present
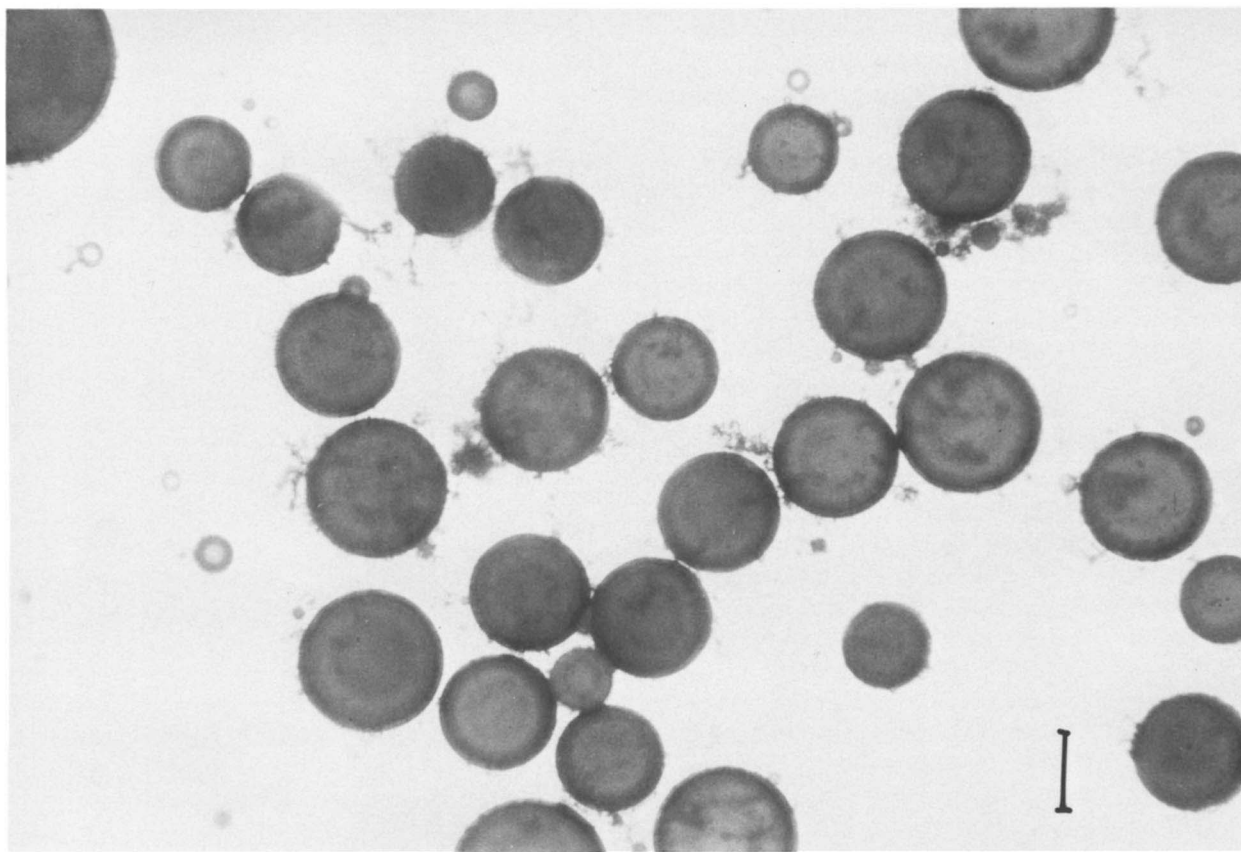
Fig. 2. Cells recovered from culture after dilution of medium, flotation of cells, and staining with oil red O. Dark contents in cells are stained red. Insert: 50 μm.

procedure, its potential usefulness for other purposes has not been tested.

Counting the cells of the epididymal fat pad revealed that the major part of the total number of cells consisted of cells other than adipocytes or those developing into adipocytes. The proportion of these nonadipocyte cells varied with rat weight and was highest in the largest rats. At rat weights of 150–200 g the adipocytes comprised 20–30% of all cells. This is a somewhat lower proportion than that obtained in the parametrial fat pad of rats of the same size (14).

Cell culture

A previous report indicated that cells developing into adipocytes are present in the epididymal fat pad from small rats (7). These cells can be cultured, forming cells of morphology and function identical with mature adipocytes. In the present work this culture medium was modified by the addition of methyl cellulose to form a medium of high viscosity, essentially preventing the cells from sedimenting and adhering to the bottom. In this medium the cells did not multiply as judged from both microscopic and biochemical studies. Counting revealed no loss of cells (see

Experimental Procedures). Thus, it is justified to assume that the number of cells in culture did not change with time during the study. Consequently, the cell countings performed at the end of the experiments were representative of the number of cells, other than mature fat cells, present in the fat pad. Since all the cells were liberated from the fat pad, the cells at the end of the Methocel cultures should be representative of all cells in the tissue save the mature adipocytes.

During culture, cells that were continuously filling up with triglyceride became evident and these cells were morphologically identical with mature adipocytes. It should then be considered from which precursor cells these adipocytes developed. It is highly unlikely that they developed from adipocyte ghosts, formed during the preparation of the cells, because deliberately prepared ghosts could not be made to develop into adipocytes. It is also highly unlikely that they developed from small mature fat cells obtained during the preparation procedures, because the cells were pelleted by a brief centrifugation at low speed and at a density that allowed the sedimentation only of cells with very little or no triglyceride. Remaining
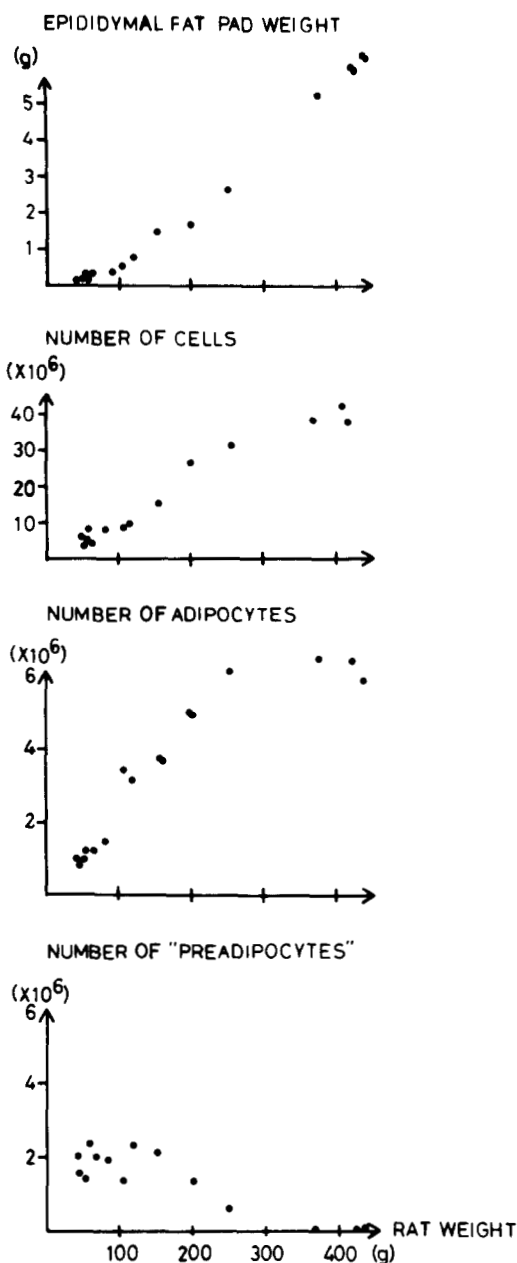
EPIDIDYMAL FAT PAD WEIGHT

NUMBER OF CELLS

NUMBER OF ADIPOCYTES

NUMBER OF "PREADIPOCYTES"

Fig. 3. Number of different cells in the epididymal fat pad of rats of different weights. Number of cells (second panel from top) was determined as follows. After incubations in enzyme solution the pelleted cells were resuspended in culture medium. In an aliquot of this suspension the total number of cells was counted in a blood-counting chamber and the total number of cells in the pellet was obtained. (For further details, see Experimental Procedures). To obtain the total number of cells in the tissue the number of adipocytes (panel 3 from top) was added. This was obtained by measuring the average fat cell diameter in the intact contralateral fat pad, and average fat cell weight could be determined. After triglyceride determination, the total number of fat cells was estimated as total triglyceride (μg)/av. adipocyte weight (μg). The number of "preadipocytes" (bottom panel) was determined as follows. An aliquot of the resuspended, pelleted cells obtained after incubation in enzyme solutions was suspended in the medium containing methyl cellulose and cultured for 3 weeks. Thereafter monovacuolar cells with a diameter >20 μm were counted as preadipocytes as described in Experimental Procedures.

pieces of tissue, containing heavy cells plus some adipocytes, were not seen in the preparations although theoretically they could have passed through the 250-μm screen. The remaining and most likely possibilities then seem to be that these cells had differentiated towards adipocytes in vivo before the rats were killed or, alternatively, that they differentiated from a precursor cell during the incubations in vitro. There seems to be no available means of distinguishing between the latter alternatives. When the cell countings were performed, however, no fat cells below a diameter of 20 μm were seen, indicating that if differentiation of cells had occurred during the culture period, this was probably terminated at the time of the cell counting. Therefore, it was assumed that all cells developing into adipocytes from different stages of isolated precursor cells were counted.

Number of different cells in the epididymal fat pad

With increasing rat weight and increasing epididymal fat pad weight, the numbers of cells other than fat cells increased and were several fold more numerous than the adipocytes at each rat weight examined. The number of adipocytes seemed to level off at a rat weight of about 200 g in accordance with the report of Greenwood and Hirsch (15). Cells developing into adipocytes (preadipocytes; for definition, see below) were present in the smaller (younger) rats where they comprised twice as many cells as the mature adipocytes. The preadipocytes then decreased in number but still constituted a significant fraction in relation to the number of mature adipocytes at rat weights of about 200 g. Above 300 g they were, however, not discovered. These results are in general agreement with those obtained in the cell cultures without methyl cellulose. Here the fraction of cells known to develop
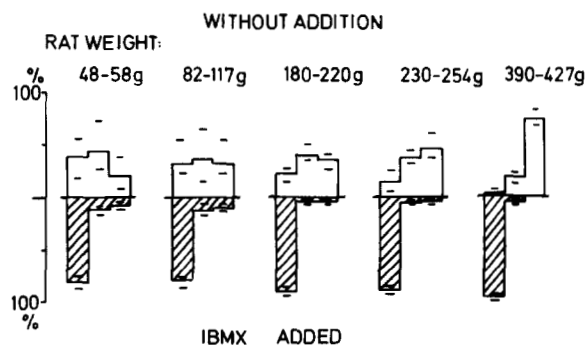

Fig. 4. Density profiles in cultures of cells from rats of different weights. Culture in medium without methyl cellulose for 5 days. Open columns, no addition; hatched columns, addition of isobutyl-methylxanthine (IBMX) at a concentration of 0.5 mM present during the whole culture period. Left column, density <1.040 g/ml; middle column, density 1.040–1.055 g/ml; right column, density 1.055–1.070 g/ml. Mean and range of three experiments.

Fig. 5. Cells from the tail skin of a 320-g rat cultured in medium without methyl cellulose for 4 weeks. Granules are red after oil red O staining. Nuclei stained by gentian violet. Insert: 50 μm.

into adipocytes (7) was the largest in the smallest rats. This fraction then decreased with increasing rat weight and appeared to disappear completely in the largest rats.

Fig. 3 reveals that in the largest rats the total number of adipocytes was about 6×10^6 and here no preadipocytes were found. At this point, then, no new preadipocytes seemed to be formed and no preformed preadipocytes were available to be filled with triglyceride. The total number of adipocytes plus preadipocytes

reaches the level of 6×10^6 at a rat weight of approximately 150 g. Before this level, then, it seems likely that formation and maturation of the preadipocytes occur until the level of 6×10^6 adipocytes plus preadipocytes is reached.

Greenwood and Hirsch (15) previously examined the problem of fat cell formation with age in the male Sprague-Dawley rat utilizing the technique of DNA labeling in vivo (6). Their conclusions were that synthesis of adipocyte precursors occurs up to the

fifth week of age, or at a body weight of about 150 g. The direct results presented here are thus in excellent agreement with this investigation. In addition, the present examinations show the quantity of the adipocyte precursors.

The results presented here are also in accordance with the current concept that no spontaneous new formation of adipocytes or preadipocytes occurs in the rat after a certain age. This concept is based on both histological evidence and DNA synthesis data (1, 15). In the presence of the potent phosphodiesterase inhibitor isobutylmethylxanthine there was, however, a pronounced formation of cells containing 30% or more of triglyceride in all groups of rats, including the largest rats, which do not show any spontaneous formation of such cells in the absence of the inhibitor.

The results reported here of the experiments in which cells were cultured without methyl cellulose must, however, be interpreted with some caution. Although there were very few cells with a content of more than 30% triglyceride in the older rats after a culture period of 5 days, one cannot exclude the possibility that such cells can be formed without additions to the culture medium under other experimental conditions. Prolonged incubations do not, however, change the picture according to additional, preliminary experiments. Furthermore, in the experiments with isobutylmethylxanthine, the lightest cells containing more than 30% triglycerides have been characterized as adipocytes by morphological and functional criteria in the small rats (7) while in the older rats, the density and morphological appearance have been the only criteria suggesting that they are adipocytes. However, this might not be sufficient as discussed below.

The results of the present report indicate, nevertheless, the important possibility that cells from the adult rat, showing no spontaneous new adipocyte formation, might have the potential to develop into new fat cells. Although under ordinary conditions in vivo, the adult, male Sprague-Dawley rat does not seem to form new fat cells in the epididymal fat pad, it may well be that new fat cells are formed under certain conditions. This is currently being studied in more detail.

The presently available methods for fat cell measurements and counting only allow observations on mature fat cells (3–5). The results presented here suggest that measurements with these methods probably are adequate when applied to tissues from adult individuals, but they also show conclusively that when used with young animals, and possibly children, these methods can give very misleading results because the number of cells for fat storage may be severely underestimated.

It was difficult to derive conclusive interpretations of the present, preliminary experiments that suggest differences in fat cell development in the rat with age. These difficulties as well as reports in the literature (16–20) demonstrate the need for distinctions, definitions, and a standardized nomenclature in this field, which apparently is now developing very quickly. The following suggestions may serve to avoid further confusion.

A cell termed an *adipocyte* should be characterized by a combination of functional and morphological characteristics. The main function of the adipocyte is to store and release triglycerides. A combination of lipoprotein lipase activity, triglyceride synthetic pathways, and hormone-sensitive lipase activity is characteristic but not specific for adipocytes and should be combined with the typical monovacuolar morphological appearance to allow the definition of an adipocyte. The lack of specificity of only one of the mentioned criteria is demonstrated by the fact that the above functional activities are present in several other types of cells, and inclusion of lipid of varying degree in cells is sometimes only a sign of malfunction.

A *preadipocyte* is a cell destined to become an adipocyte. It could be at any stage of the lipid-filling process but should have the same functions as a mature adipocyte. A demonstration that the cell is developing into a mature adipocyte should also be required before it is labeled a preadipocyte because of the relative unspecificity of the functional characteristics, particularly lipid granulation.

We also suggest that in developmental stages before the preadipocytes, cells not yet identified as developing into adipocytes should be labeled *adipoblasts*. It is logical that the definition of these cells should require the demonstration that they form adipocytes via preadipocytes as defined above.

Other cells that contain lipid but do not satisfy the functional criteria mentioned and do not have or develop the typical morphology of a mature, monovacuolar adipocyte might tentatively be labeled by the descriptive term *lipocytes* if they cannot be characterized as other well-defined types, such as endothelial cells or smooth muscle cells, for example.

The present and previous (7) studies allow the described cells to be defined according to these criteria. Thus it has been shown that adipocytes indeed develop from a cell fraction that is isolated and purified from the epididymal fat pad of small rats. After the formation of a confluent monolayer, this cell fraction divides and develops into cells with the functional characteristics of adipocytes and with increasing lipid gran-

ulation. These cells develop into adipocytes; they therefore fulfill the criteria of being adipoblasts while in the multiplying phase and preadipocytes after confluence. This definition also allows the conclusion that adipoblasts lack important functional characteristics of preadipocytes and adipocytes (7) that evidently develop at the stage of monolayer confluence. Presumably the cells shown to develop into adipocytes in methocel-containing culture medium in the present work are also preadipocytes or, possibly, adipoblasts, but a full definition is still lacking because the functional criteria cannot be determined without a method for the isolation of these cells. Descriptively, however, they do develop adipocyte morphology and, since they are prevented from multiplication, they probably give a measure of the number of preadipocytes present in vivo. It cannot be ruled out conclusively that adipoblasts present in vivo are also included here but, since these cells lack the functional equipment to accumulate lipid (7 and discussion above), it must then be assumed that they develop from adipoblasts to preadipocytes during the procedures in vitro.

The cells described in the present experiments with culture in Methocel-free media have been defined as adipoblasts, preadipocytes, and adipocytes in the previous work (7) as far as the group of smallest rats is concerned. With the older rats, however, the criteria suggested above are only partially fulfilled; therefore these cells, by definition, are lipocytes until further characterization has been established. This reasoning is also applicable to cells that have been described previously in the literature where the functional criteria are partially fulfilled but full functional and morphological definition is lacking (17–19), or where the only basis for definition has been lipid granulation (16, 20).

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