Cellular and vascular development in immature rat adipose tissue

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Abstract Techniques that allow the study of immature subcutaneous depots in newborn and young rats were developed utilizing a histological and histochemical approach. The temporal and spatial relationship between blood vessel development and adipocyte hypertrophy was examined in rats from large and small litters. Large numbers of small (9.8 ± 1 μm diameter) multilocular adipocytes were present in subcutaneous depots of newborn animals. These adipocytes were intimately associated with capillaries that had lumen diameters of 4–10 μm. Adipocytes rapidly increased in size up to 30–40 μm in diameter. The development of small capillaries (2–4 μm lumen diameters) was associated with this cell hypertrophy. Lipoprotein lipase (LPL) histochemistry and ultrastructural analysis indicated that the larger capillaries may potentiate LPL-mediated cell hypertrophy. Similar analysis indicated that the development of the narrow lumen capillaries may slow the rate of LPL-mediated hypertrophy. These studies indicate that the adipose tissue capillary may play an important role in adipocyte hypertrophy and metabolism.—**Hausman, G. J., and R. L. Richardson.** Cellular and vascular development in immature rat adipose tissue. *J. Lipid Res.* 1983. **24:** 522–532.

Supplementary key words lipoprotein lipase

The small amount of body fat (1%) in the newborn pig and rat (1) could be interpreted as evidence that the rat and pig fetus have little or no adipose tissue. Adipose depots in the fetal pig and rat are difficult to locate and dissect out because of their small size and low lipid content. Immature depots can be identified and studied with histological techniques. Research has indicated that the fetal pig contains a large number of small adipocytes (2). Preliminary work in our laboratory indicated that the newborn, unsuckled rat also contains a large number of small adipocytes (3). Therefore, the fetal stage of pig and rat adipose tissue development is achieved primarily by an increase in adipocyte number. The study of adipose tissue in the fetal pig and rat would yield information regarding the immature adipocyte. We have developed procedures that allow the study of immature subcutaneous depots in longitudinal age studies of fetal pigs and young rats.

Changes in lipoprotein lipase (LPL) activity per adipocyte precede significant changes in fat cell size in the developing epididymal rat fat pad (4). Hietanen and Greenwood (4) suggested a regulatory role for lipoprotein lipase in the control of adipocyte lipid filling during early development. Other biochemical studies extended these findings (4) to other depots in the young rat (5, 6). These biochemical studies have not established the temporal and spatial relationships between LPL development and adipocyte and capillary development. Techniques for the cytochemical localization of LPL activity have been described (7). We have investigated the temporal and spatial relationship between the development of capillaries and adipocyte hypertrophy by cytochemical localization of LPL in sections of immature rat adipose tissue. Litter size was varied to determine the effect of over- and undernutrition on the histochemical and morphological development of capillaries.

MATERIALS AND METHODS

All animals were Sprague-Dawley rats supplied by the Charles River Company. They were fed TEK-LAD (Winfield, IA) rat chow ad libitum and housed in a temperature-controlled room with a 12-hr light–dark cycle. Two rats per litter from a total of 25 litters were killed; they ranged in age between newborn, unsuckled animals to 28-day-old animals. To obtain small litters (<8), litters of 10 were adjusted at birth. Large litters (>10) were natural litters; cross-fostering was not done. Rats were killed by decapitation.

Tissue handling

Young rats (<5 days) were cut into three equal-sized cross sections (cranial, middle, and caudal). One cut was made near the umbilicus and the other was made caudal to the shoulders. The front and back legs and tail were removed from the sections. For enzyme analysis, the sections were frozen in isopentane cooled in liquid nitrogen and stored at −60°C. To obtain paraffin sec-

Abbreviation: LPL, lipoprotein lipase.

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tions, the body sections were placed in Bouin's fixative for 12–18 hr. The sections were removed and sliced into 1 to 3 mm whole-body cross sections. The sections (1–3 mm) were fixed for an additional 24 hr in fixative and then routinely processed into paraffin blocks.

Section preparation and staining

Mounting of body sections in paraffin blocks or the cryostat was done so histological sections were perpendicular to the skin surface. Specific procedures for handling and staining fresh frozen cryostat sections were reported elsewhere (8). Paraffin sections (5–10 μm) were stained with the periodic acid-Schiff (PAS) reagents (9), the picro ponceau reagents (9), Lillie's Allochrome reagents (10), and Harris Hematoxylin (HH). Air-dried, fresh frozen cryostat (−20°C) sections were stained with oil red O (8), the picro ponceau reagents (9), toluidine blue (9), and with procedures for alkaline phosphatase (20-min cold acetone fixation, 9), ATPase (11), and LPL (7).

Electron microscopic analysis

Light-microscopic analysis revealed that the rate of subcutaneous adipocyte hypertrophy was maximum at 2 days of age regardless of litter size. In efforts to further resolve cellular and vascular relationships, samples of inguinal adipose tissue were obtained from each of two rats at 2, 10, and 28 days of age. Small blocks of tissue were immersed for 1 hr in cold 2% gluteraldehyde buffered with Sorenson's phosphate (pH 7.2). Postfixation for 1.5 hr was in cold 1% osmium tetroxide solution buffered with Michaelis veronal acetate (pH 7.4). The blocks were dehydrated in a graded ethyl-alcohol series. Epon[1] was used to embed the samples. Thin sections (silver) were placed on 200-mesh grids and stained for 8 min with 0.5% uranyl acetate and 5 min with 0.4% lead citrate. Grids were examined with a Hitachi HU-12 electron microscope.

Fat cell size

Adipocyte diameters were determined on paraffin sections according to the method of Sjöström, Björntorp, and Vrána (12). Fifty cells were measured per animal.

RESULTS

Fig. 1 contains growth curves for four litters of various sizes. There was little difference in the growth of

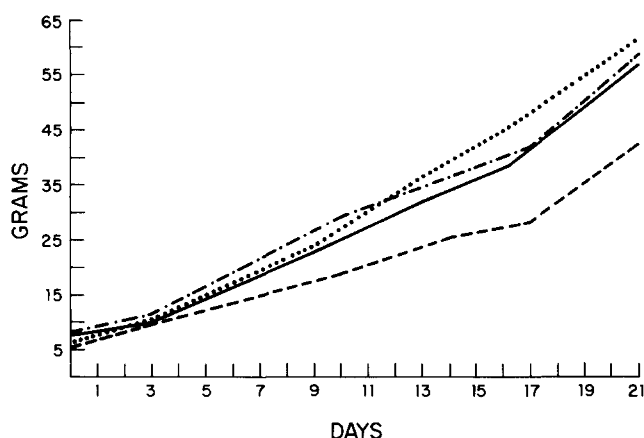

Fig. 1. Growth curves for rats reared in litters of six (· · ·), ten (–·–·–, ——), and eighteen (– – –). Each curve represents the average pup weight of a single litter determined at birth, and at 3, 10, 14, 17, and 21 days of age. The litter with six pups (· · ·) contained twelve pups until day 3, at which time six pups were removed from the litter.

rats from litters of six and ten. Rats raised in litters of 18 grew at a much slower rate. The average birth weight of rats from various litter sizes was 7.1 ± 0.8 grams (mean ± SD of 25 rats).

Identification and characterization of immature subcutaneous adipose depots and adipocytes

Maintenance of a constant sectioning plane (cross section with respect to the long axis of the body) throughout tissue and section preparation permitted the identification of specific areas of subcutaneous tissue from newborn and young rats. The shape of the whole body cross section and mammary gland ducts and lymph nodes (located dorsal to the back legs) were used to identify the immature inguinal adipose pad (**Fig. 2**). Inguinal adipocytes were in histological sections that contained or were close to proximal aspects of the back legs (Fig. 2). A characteristic of these sections was a general shape that was almost rectangular (Fig. 2). Generally, adipocytes were not adequately outlined in lipid-stained cryostat sections (**Fig. 3**) to allow accurate measurement of diameter. The low lipid content and the multilocular morphology made it difficult to resolve the cell borders (Fig. 3). Adipocytes were clearly outlined in PAS- and Lillie's Allochrome-stained paraffin sections from newborn and rats of all ages (**Fig. 4**). In these sections, diameters of adipocytes could be accurately measured and macrophages, fibroblasts, and other cells could be distinguished from adipocytes. Adipocytes in newborn, unsuckled rats were consistently multilocular (many lipid droplets) and were 9.8 ± 1 μm in diameter (mean ± SD of 20 rats from a total of 10 litters, i.e., 2/litter). Litter size had no effect on cell size in newborn rats.

[1] Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee of warranty by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products.

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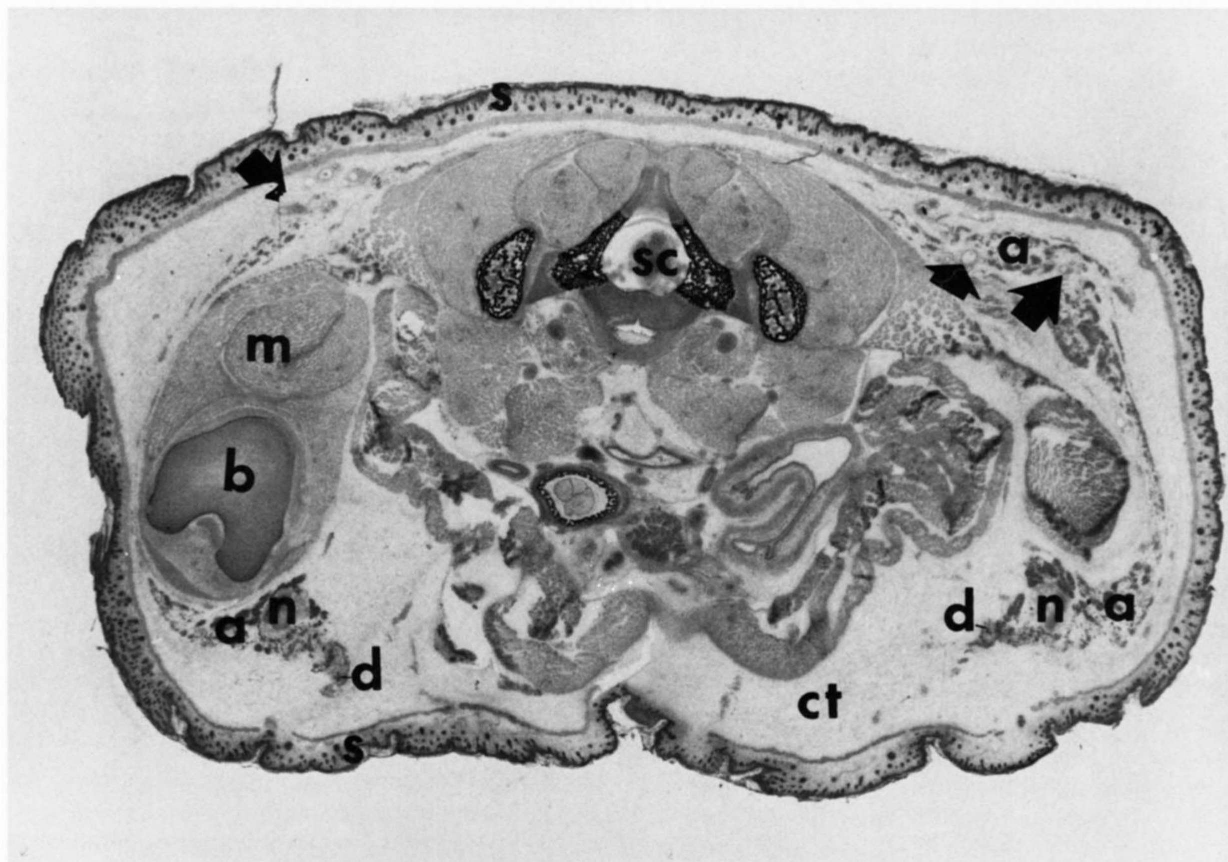

Fig. 2. Whole body cross section through the inguinal area of a newborn male rat. Note the large numbers of lipid containing adipocytes (a) around mammary gland ducts (d), mammary gland lymph nodes (n) and blood and lymph vessels (arrows). Note the approximate rectangular shape of the section. Cryostat section (24 μm) stained with oil red O (lipid) and HH, ×10. Also indicated are spinal cord (sc), muscle (m), skin (s), and lipid-free connective tissue (ct).

Adipocyte hypertrophy

Fat cell hypertrophy during the first 3 days of life was dependent on litter size (**Table 1**). Cell diameters nearly tripled in rats from small litters (Table 1). In rats from the largest litters cell diameters nearly doubled (Table 1). In all the 3-day-old rats the majority of adipocytes (>95%) were unilocular (one central lipid droplet). Fat cells in 10-day-old rats were 37 ± 8 μm in diameter (mean ± SD of six animals, equally representing large and small litters).

Blood vessel histology

Capillaries in adipose tissue of animals less than 24 hr old were very tortuous and extensively branched (Fig. 4B). Analyses of 10 to 20 serial sections per animal from ten newborn animals indicated that most adipocytes were part of large clusters of tightly packed cells and capillaries (Fig. 3). Many of these clusters were larger than 100 μm by 100 μm. Capillaries were extensively distributed in these large adipocyte clusters and the space between adjacent capillaries was completely filled by adipocytes (Fig. 4). Many nuclei were associated with capillary walls and lumen diameters ranged between 4–10 μm. Adipocytes larger than 30 μm were associated with straighter capillaries that had lumen diameters of 2–4 μm and fewer associated nuclei (**Fig. 5**). In animals where most (>95%) of the adipocytes were larger than 30 μm, the predominant capillary had a lumen diameter of 2–4 μm. This decrease in capillary lumen diameter (associated with adipocyte hypertrophy) was not associated with a change in the relative concentration of small arterioles and venules (Fig. 5).

In comparison of small and large litter rats, the small capillaries (2–4 μm) developed at an older age in the large litter rats.

Blood vessel histochemistry

Capillaries in newborn rats did not stain for ATPase or LPL activities (**Fig. 6**). In young rats (12–72 hr) from small litters large capillaries (4–10 μm lumen diameters) were uniformly and intensely stained for LPL activity (Fig. 6). In large litter rats of the same age, there was

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little capillary LPL staining (Fig. 6). Regardless of litter size, capillary LPL staining was most intense in the large adipocyte clusters (Fig. 6). In animals where most of the adipocytes (>95%) were larger than 30 μm, capillaries with lumen diameters of 4 μm were lightly stained (LPL) whereas capillaries with lumen diameters of 2 μm did not stain (**Fig. 7**). In comparison of large and small litter rats, capillary LPL staining was observed at earlier ages in small litter rats.

In mature depots (>30 μm adipocyte diameters), capillaries (2–10 μm) were lightly stained for ATPase activity. Arterioles and larger arteries were stained for alkaline phosphatase and ATPase activity in all ages studied. Capillaries did not stain for phosphatase activity at any age.

Ultrastructure of capillaries in 2-day-old rats

Capillary walls were thick and contained many organelles (**Fig. 8**). Endothelial cytoplasm contained mitochondria, ribosomes, rough endoplasmic reticulum, glycogen, microtubules, microfilaments, and many ves-

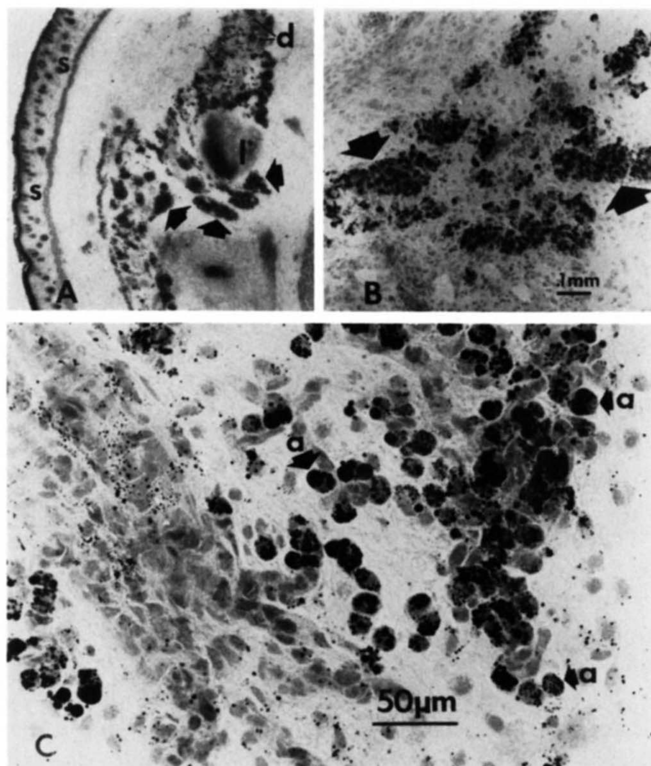
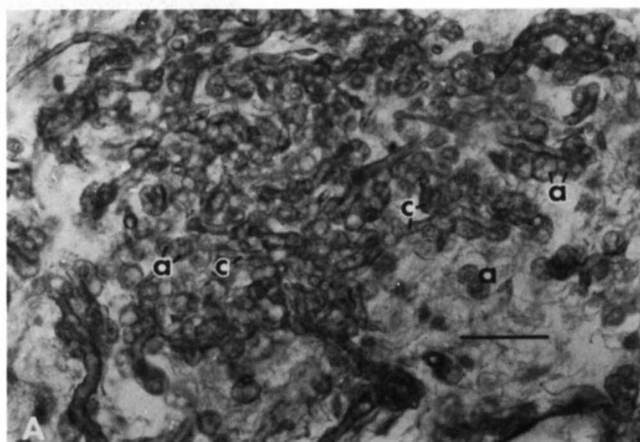
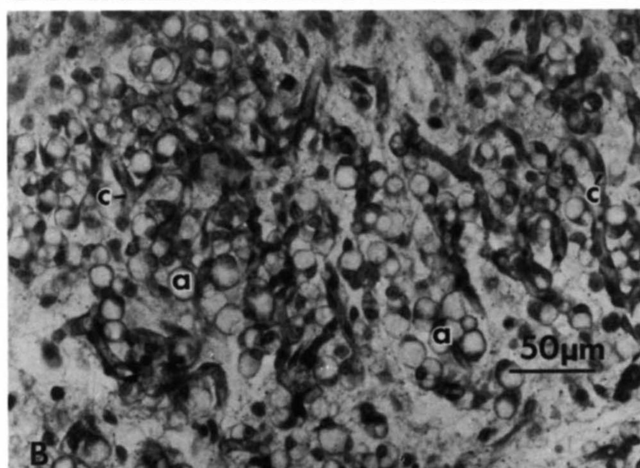


Fig. 3. Lipid-containing adipocytes (C, a) and fat cell clusters (A, B, arrows) in a section from a newborn, unsuckled rat. Fat cell clusters (A, arrows) around lymph nodes (l) are much larger than clusters around mammary gland ducts (d). Most adipocytes are arranged as large clusters of tightly packed cells (B, arrows). The multilocular nature of the cells (a) is depicted in C. In lipid-stained sections, adipocyte-to-adipocyte distinction is minimal where adipocytes are tightly clustered. Cryostat sections (24 μm) stained with oil red O (lipid) and HH.





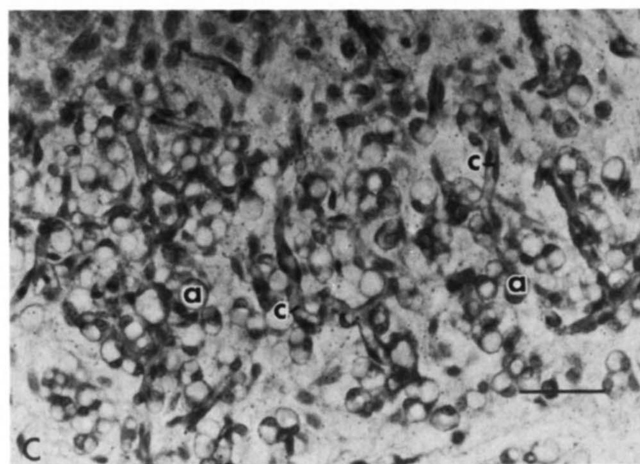


Fig. 4. Adipocytes (a) and capillaries (c) in a section from a newborn, unsuckled rat (A) and in serial sections from an 18-hr-old rat (B and C). In Lillie's Allochrome-stained sections (A), individual adipocytes (a) can be distinguished in large cell clusters due to the staining of the external lamina surrounding the cells. Note the large number of capillaries (c) and adipocytes (a) that comprise the large fat cell clusters. (A, B, and C), all the capillaries in these sections have lumen diameters greater than 4 μm. Sections from 18-hr-old rats are shown (B and C) because capillaries are better demonstrated due to slightly larger adipocytes (a). The use of serial sections (B and C) demonstrates the extensive nature of the capillaries and the clustering of adipocytes (a) around the capillaries (c). Paraffin sections (10 μm) stained with Lillie's Allochrome reagents (A) or with PAS reagents and HH (B and C).

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TABLE 1. Fat cell diameters and body weights of 3-day-old rats from litters of various sizes

Fat Cell Size	Litter Size	Body Weight
μm		g
33.6 ± 0.4[a]	2	10.6 ± 0.07[b]
31.7 ± 1	6	14.65 ± 0.4
29.5 ± 1	6	9.9 ± 0.3
27.5 ± 0.6	8	10.6 ± 0.0
24.6 ± 0.3	10	9.8 ± 0.9
22.6 ± 0.6	13	9.4 ± 0.2
20.8 ± 0.4	15	8.7 ± 0.14
19.7 ± 0.3	17	8.8 ± 0.8

[a] The mean ± SD of the average fat cell size (n = 50) from each of two animals/litter.
[b] The mean ± SD of the weight of the same two animals/litter used for fat cell size determinations.

icles (~700 nm in diameter) that in some instances were present as complex three-dimensional arrangements, opening to invaginations of the plasma membrane (Fig. 8). Finger-like projections of endothelial cell cytoplasm into capillary lumina were frequently observed (Fig. 8). External laminae around capillaries were thick and diffuse (Fig. 8).

Ultrastructure of capillaries in 10-day-old rats

Relative to 2-day-old rats, capillary walls were much thinner and contained fewer organelles (**Fig. 9**). There were numerous finger-like extensions of endothelial cell cytoplasm into vessel lumina and the extravascular space (Fig. 9). Serial section analysis indicated that cell projections in the extravascular space extended very close to adipocytes (Fig. 9). As in younger rats, external laminae were diffuse and thick (Fig. 9).

Ultrastructure of capillaries in 28-day-old rats

Endothelial cytoplasm contained very few organelles and capillary lumina were extremely narrow (Fig. 8). An ultrastructural characteristic not shared by capillaries in younger animals was the presence of large numbers of very small pinocytotic vesicles (~350 nm) (Fig. 8). In many instances, these vesicles were preferentially located along the capillary plasma membrane closest to an adipocyte. External laminae were distinct, continuous, and thin (Fig. 8).

DISCUSSION

This study represents the first qualitative and quantitative analysis of capillaries and adipocytes in a developing, immature (all adipocytes < 20 μm) depot. Utilizing techniques that permitted the identification of adipose depots in the newborn rat, we were able to characterize and study a very early phase of adipocyte and adipose depot development. The results presented clearly demonstrate that adipocyte hypertrophy is a predominant feature of subcutaneous adipose tissue development in the young rat. We recorded a phenomenal rate of adipocyte hypertrophy in rats from small litters. Cell size data from older animals indicated that the rate of cell hypertrophy slows down considerably. The extensive vascularity in the adipose depots in the newborn rat indicated that fluctuations in blood flow could be responsible for the variations in rates of cell hypertrophy.

Histological, histochemical, and ultrastructural data indicate that large capillaries (>4 μm lumen diameter) may allow maximum uptake of lipid substrates by potentiating the action of LPL. Red blood cell and substrate flow would be physically unimpeded in large lumen capillaries. The tortuous course of the large capillaries and the close spatial relationship of adipocytes to capillaries results in minimal distance for LPL movement from adipocyte to capillary lumen and for fatty acid movement from lumen to adipocyte.

The histochemical technique for LPL activity utilizes endogenous chylomicrons and very low density lipoproteins (VLDL) for substrates (7). Therefore, reaction product deposition indicates sites of chylomicron and VLDL deposition as well as sites of enzymatic activity. Large capillaries were uniformly and intensely stained indicating large concentrations of substrates along the entire length of adipocyte-associated capillary. Lighter staining for LPL was associated with a decreasing lumen diameter to the point that capillaries ≅ 2 μm in diameter did not stain. Therefore, small capillaries may not be involved in LPL-associated adipocyte hypertrophy to the same extent as large capillaries (>4 μm lumen diameter).

Our ultrastructural data also support the concept that large capillaries may accentuate LPL-associated adipocyte hypertrophy. If the transport of LPL from adipocyte to capillary is energy-dependent (13), then the high organelle content of the large capillary walls would favor enzyme transport. The very small capillaries (≅2 μm) contained few organelles and therefore may not be as capable of energy-dependent transfers.

There was no histological evidence that new blood vessels were formed in adipose tissue of the young rat. The relative number of arterioles and venules did not change in the first few days of life. Therefore, the vessels between arterioles and venules (capillaries) become more extensive in the early postnatal period. The "larger capillary bed" was primarily due to longer and narrower capillaries. Thus, adipocyte hypertrophy and maturation of capillary beds represent two components of adipose depot expansion.

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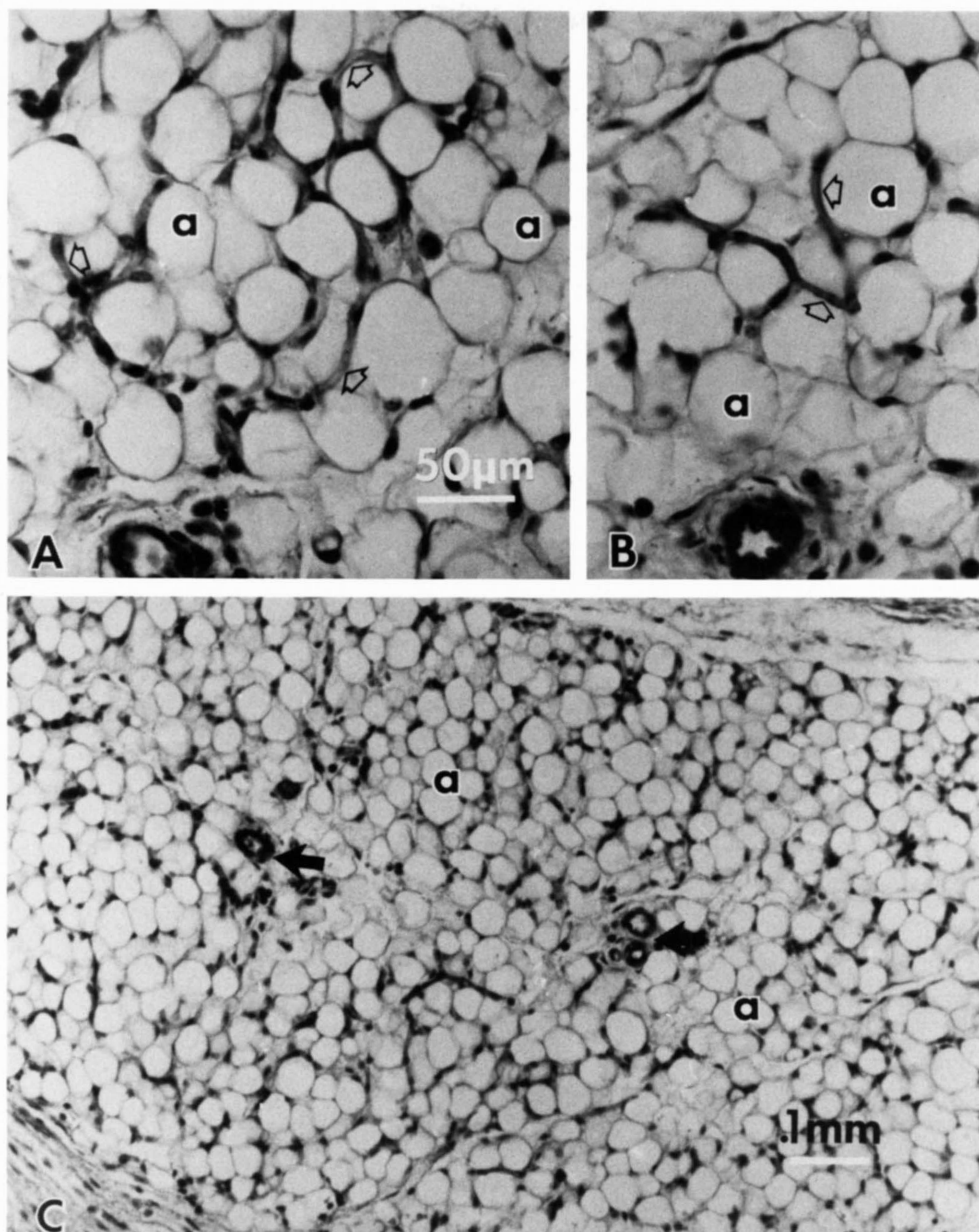

Fig. 5. Adipocytes (a) from a 3-day-old rat raised in a litter of six (A, B, and C). Compared to capillaries in younger rats (Fig. 4), capillaries in these sections (A, B, arrows) have smaller lumen diameters (≤4 µm) and increased distance between endothelial nuclei. The relative concentration of small arterioles and venules (C, arrows) is shown and is unchanged from younger rats. These figures demonstrate that the vascular bed becomes very extensive as adipocytes hypertrophy after birth. Paraffin sections (10 µm) stained with the PAS reagents and HH. Linear scale in A also applies to B.

Our results are compatible with a report on LPL distribution in adipose tissue of (50–700 g) larger rats (14). In four depots, the activity of LPL per 10^6 cells increased with increasing cell size (14). Increasing cell size was also associated with an increased percentage of whole tissue LPL contained in adipocytes (14). This "decreased extracellular localization of LPL" was paralleled in vivo by decreased uptake of chylomicron triglyceride fatty acids by larger adipocytes (14). A logical assumption extending from our results would be that this decreased functionality of LPL (14) could be associated with an increased percentage of very small (2 µm) capillaries.

Plasma membrane-associated vesicles were larger and

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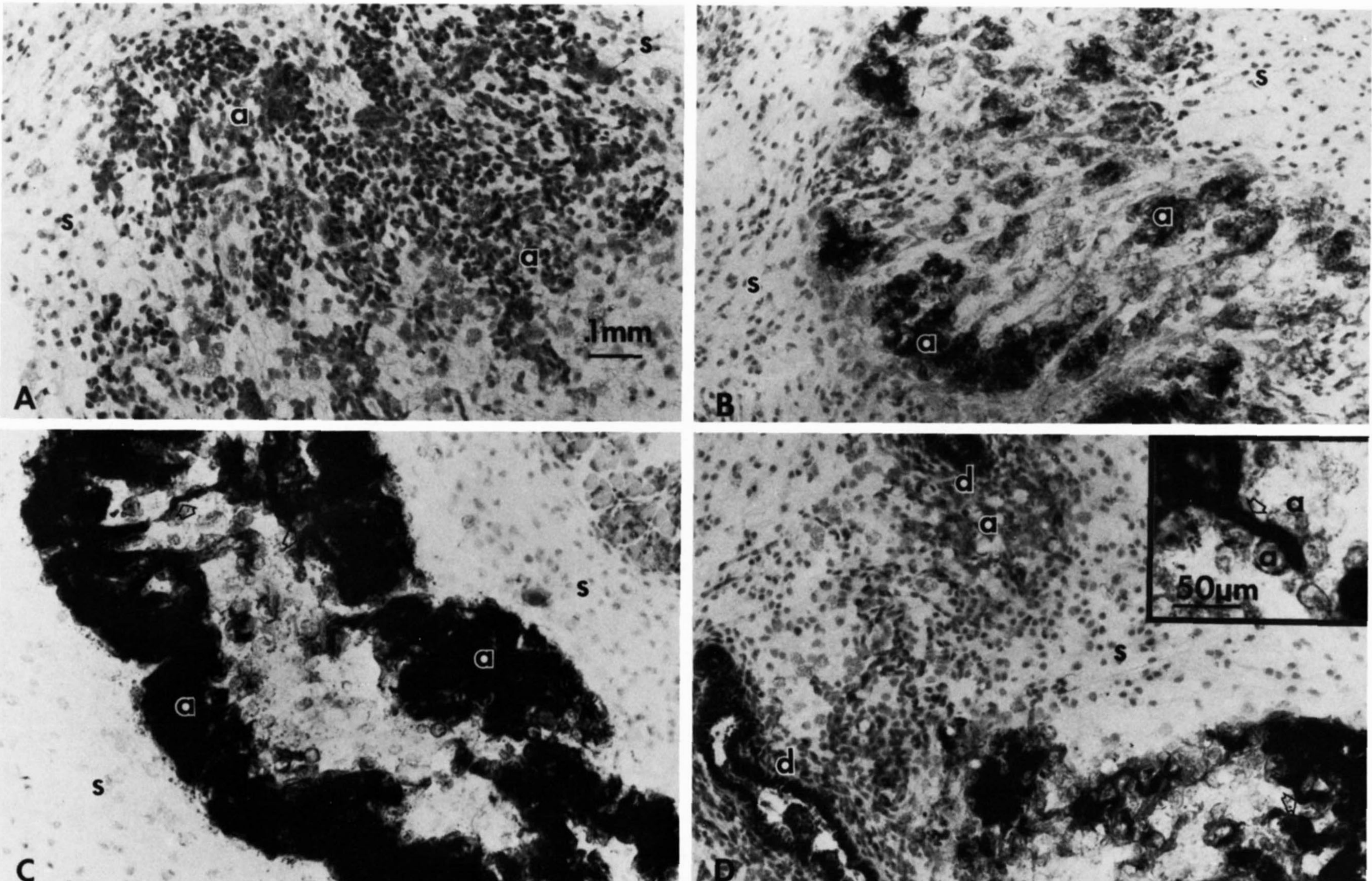

Fig. 6. Sections reacted for lipoprotein lipase activity from a newborn unsuckled animal (A), a 30-hr-old animal from a litter of 15 (B), and a 30-hr-old animal from a litter of 6 (C and D). Note the absence of reaction product in adipocyte clusters (a) in the newborn animal (A) compared to the intense staining of adipocyte clusters (a) in the 30-hr-old animal from a small litter (C). Intermediate staining is noted in an animal from a large litter (B). The intense staining in C is localized in capillaries (arrows in C and D and in inset) that have lumen diameters greater than 4 µm. Adipocyte clusters (a) around mammary gland ducts (D, d) are smaller and show little stain. D and C are from the same section. Linear scale in A applies to B, C, and D. All sections reacted for LPL for 4 hr at 37°C and then counterstained with HH. Also indicated are stromal areas (s).

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more organized in capillaries from 2- and 10-day-old rats than in capillaries from 28-day-old rats. Rosettes are complex structural arrangements of adipocyte plasma membrane vesicles, considered to be important in fatty acid egress from the cell (15). Similar structures in the young rats were probably involved with fatty acid transport across the capillary wall, considering the considerable LPL-associated cell hypertrophy. Since the older rats (28-day-old) are exposed to much less preformed dietary lipid, the smaller capillary vesicles (older rats) may be associated with glucose transport.

Capillaries of brown adipose tissue (BAT) are stained intensely when tested for alkaline phosphatase activity (16). Our results with alkaline phosphatase are in accord with a previous study that demonstrated no activity in white adipose tissue capillaries (16). Muscle capillaries are intensely stained when tested for ATPase activity.[2] ATPase staining of adipose capillaries may indicate a metabolic maturity similar to that in muscle capillaries.

Our results of the effects of litter size on adipocyte size and LPL activity are in general agreement with a study of slightly older animals (5). As in our study, a plateau in adipocyte hypertrophy was achieved at an earlier age for small litter rats (5).

Recently, a model for the transfer of lipid across capillary endothelium has been proposed (17). The central theme of the proposal entails the location and lateral movement of lipolytic products in a continuous interface composed of the chylomicron surface film and the external leaflet of plasma and intracellular membranes of endothelial and parenchymal cells (17). This theory presupposes that at some point the external leaflets of plasma membranes of endothelial cells and adipocytes or pericytes are fused (17). Our data did demonstrate projections of adipocytes and capillaries in close proximity but did not confirm fusion of external leaflets of plasma membranes of these cells. In the 2-day-old rats, the predominant ultrastructural aspect of capillary plasma membranes were vesicles arranged as rosettes (15). Numerous "rosettes" were observed in cultured 3T3-L1 preadipocytes during differentiation (18). Therefore, rosettes are not involved in fatty acid transport via continuity of plasma membrane external leaflets. Complex arrangements of adipocyte surface-associated vesicles have been implicated in potentiating the action of insulin by providing more interaction of cytoplasmic elements with the cell surface (19). In this regard, our data would indicate that the capillary endothelial cells are principle sites of insulin action. Further studies of adipose tissue capillaries are needed to confirm this possibility.

[2] Hausman, G. J. Unpublished observation.

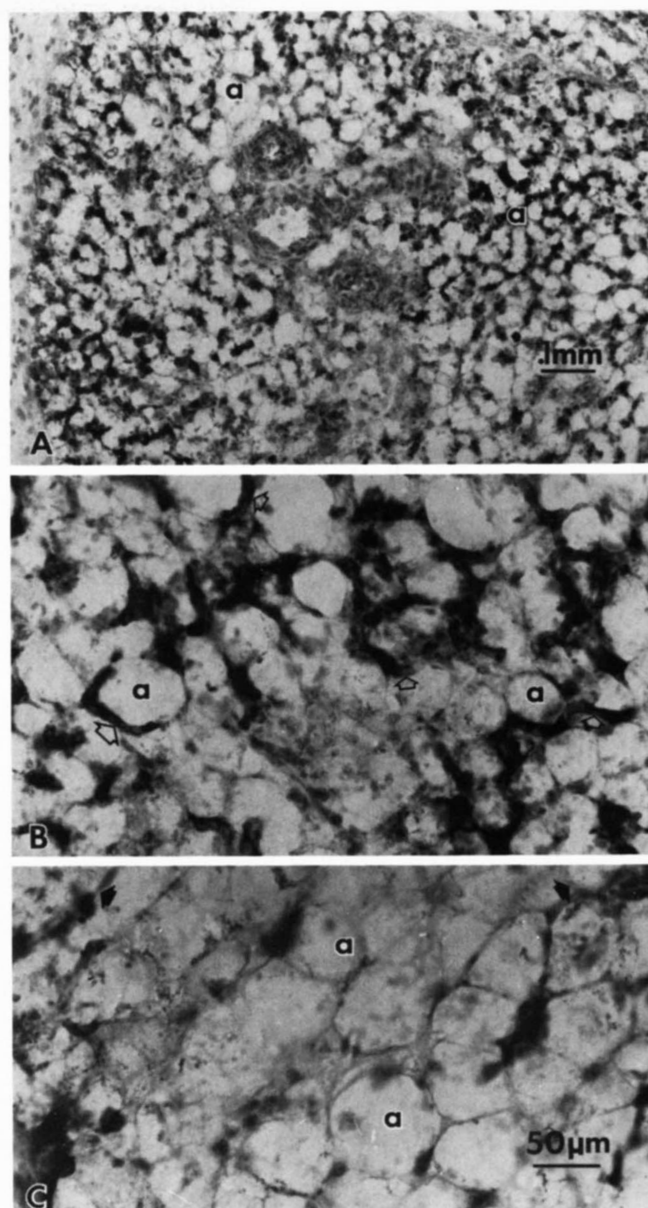

Fig. 7. Sections reacted for LPL activity from a 10-day-old animal reared in a litter of 11 pups (A and B) and from a 7-day-old animal reared in a litter of 6 (C). Note the generalized reduced reaction product (A) in these animals compared to younger animals (Fig. 6). The capillaries were smaller and lightly stained (B, arrows) or not stained at all (C, arrows). Capillaries indicated in B (arrows) are approximately 4 μm in diameter whereas capillaries indicated in C (arrows) have lumen diameters of 2 μm. Inset scale in C applies to B also. Frozen sections reacted for LPL for 4 hr at 37°C and then counterstained with HH. Indicated are adipocytes (a).

CONCLUSION

Adipocytes in newborn rats (9.8 μm) rapidly hypertrophy to 20–35 μm (3 days). Large capillaries (4–10 μm) were only associated with small adipocytes (<30 μm in size). Larger cells (>30 μm) hypertrophy at a

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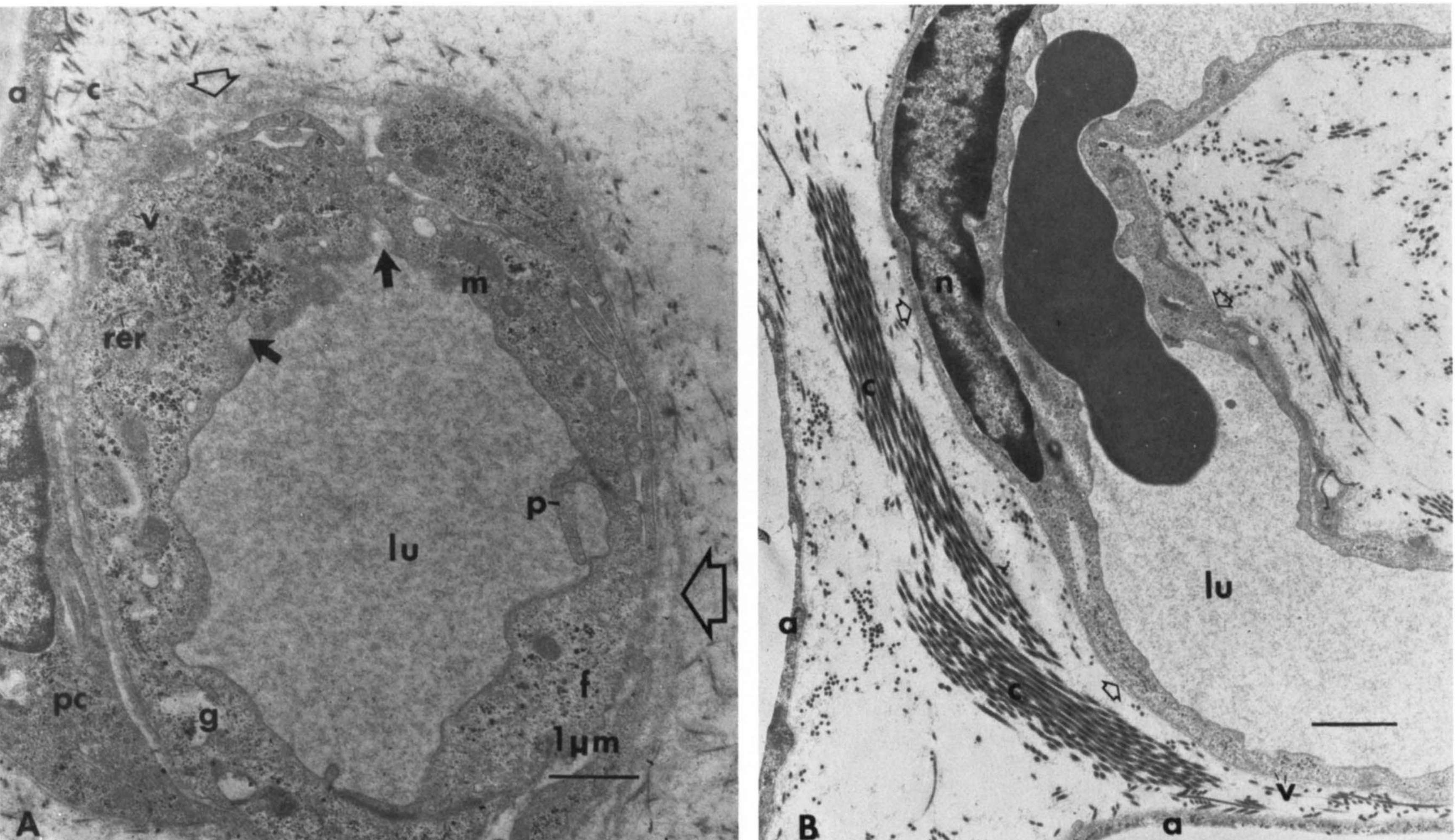

Fig. 8. Capillaries in adipose tissue from a 2-day-old rat (A) and from a 28-day-old rat (B). The lumen diameter of capillary in A is greater than 4 μm. Note the thick vessel walls (A) surrounding the lumen (lu). Capillary cytoplasm (A) contains rough endoplasmic reticulum (rer), glycogen (g) microfibrils (f), mitochondria (m), and numerous vesicles (v) that in some instances were arranged as "rosettes" around invaginations of the luminal plasma membrane (closed arrows). A thick and irregular external lamina (open arrows) surrounds the capillary. Finger-like cell processes (p) projected into the lumen. Capillaries in older rats (B) have thinner walls and a narrower lumen ($\simeq 2$ μm). Capillary cytoplasm (B) contained numerous vesicles (v) that were half the size of vesicles in younger rat capillaries (A). The external lamina (arrows) surrounding the older capillaries was thin, distinct, and continuous. Inset scale in both A and B is equal to 1 μm. Also indicated on the micrographs are adipocytes (a), collagen (c), nucleus (n) of endothelial cell, and perivascular cell (pc).

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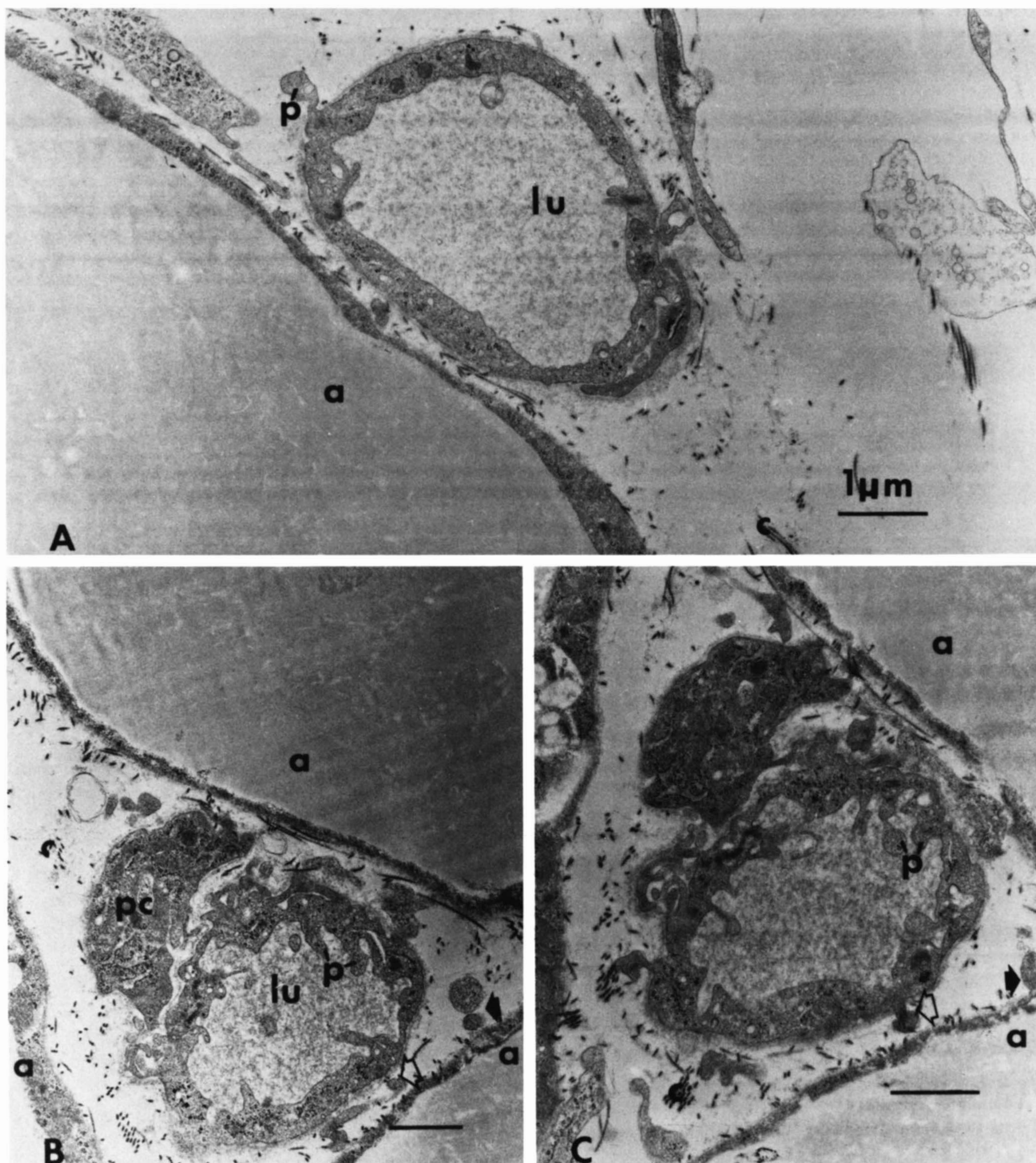

Fig. 9. Capillaries in adipose tissue from 10-day-old rats (A, B, and C). Narrow lumen (lu) capillaries (2–3 μm) are closely associated to adipocytes (a). Note that the thinnest layer of adipocyte cytoplasm is in the area near the capillary in A. The capillary walls are thinner and contain few organelles. B and C are serial sections shown here to demonstrate that cell processes in the extra vascular space (open arrows in B and C) may project towards adipocytes. The closed arrows in B and C indicate an adipocyte process that may lie close to a capillary process. This capillary (B and C) is deeply invaginated and has many cell processes (p) projecting into the lumen. Inset scale is equal to 1 μm in A, B, and C. Also indicated are collagen (c) and perivascular cell (pc).

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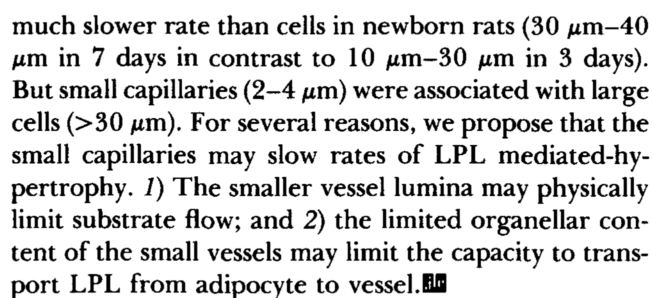

much slower rate than cells in newborn rats (30 μm–40 μm in 7 days in contrast to 10 μm–30 μm in 3 days). But small capillaries (2–4 μm) were associated with large cells (>30 μm). For several reasons, we propose that the small capillaries may slow rates of LPL mediated-hypertrophy. *1*) The smaller vessel lumina may physically limit substrate flow; and *2*) the limited organellar content of the small vessels may limit the capacity to transport LPL from adipocyte to vessel.

Manuscript received 31 March 1982 and in revised form 6 January 1983.

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