Int. J. Dev. Biol. 36: 265-274 (1992)

Original Article

Brown adipose tissue as a derivative of mesoderm grafted below the kidney capsule. A model for differentiation of isolated rat mesoderm

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ABSTRACT **During development, mesoderm differentiates into connective tissue, cartilage, bone, muscle and kidney. In experimental conditions the developmental spectrum of mesoderm grafted below the kidney capsule is reduced so that mostly brown adipose tissue (BAT) appears. Since BAT is a particular tissue with a specific developmental pattern, the structural and functional characteristics of experimentally developed BAT were analyzed in the present study. Mesoderm from nine-day-old rat embryos was grafted below the kidney capsule of adult rats and one month later the BAT-containing tumors were analyzed. The ultrastructural and morphometrical data of BAT-containing tumors were the same as in BAT developed *in situ*. Tissue-specific mRNA for uncoupling protein (UCP) was expressed in BAT-containing tumors, and immuno-electron microscopical analysis showed that mitochondria of these brown adipocytes contained UCP. Injections of noradrenaline and exposure of BAT-tumor-bearing rats to cold stress increased both the amount of UCP and the expression of UCP mRNA in tumors of BAT; i.e., experimentally developed BAT entirely resembled standard BAT. It is proposed that mesoderm isolated and displaced below the kidney capsule lacks the inductive stimuli of ectoderm and endoderm, and as a result mesoderm can not realize the natural pattern of differentiation. Here, in a new environment, mesoderm is exposed to new inductive stimuli which induce differentiation of mesoderm into BAT, probably through neuro-vascular elements from the medial side of the kidney (BAT area). Thus, although mesoderm contains a wide differentiation capacity, it can differentiate into only one type of tissue, depending on the presence and range of inductive stimuli.**

KEY WORDS: *mesoderm, brown adipose tissue, uncoupling protein, mitochondria, differentiation, rat*

Introduction

With an exact anatomical location and structure (Afzelius, 1970) and a unique thermogenic function (Nicholls and Locke, 1984), brown adipose tissue (BAT) is a particular kind of connective tissue present in small rodents and hibernators. BAT together with convertible adipose tissue (Loncar and Afzelius, 1989; Loncar, 1991a,b) belongs to the group of thermogenic adipose tissue, i.e., adipose tissue that has the capability to produce heat, and in this way, differs from the nonthermogenic adipose tissue, known as white adipose tissue (WAT).

During the perinatal period, during arousal from hibernation or during exposure to a cold environment, mitochondria in thermogenic adipocytes produce mostly heat instead of ATP. Cristae of these mitochondria contain a 32 kDa protein known as the uncoupling protein (UCP) (Ricquier and Bouillaud, 1986). UCP permits those protons which were pumped during the oxidation process into the intermembranous space to return into the mitochondrial matrix through channels made from UCP dimers rather than through ADP/ATP-ase (Nicholls *et al.*, 1986). In this process the heat is released. A highly developed vascular system disseminates this heat over all the body (Foster, 1986). Since among all analyzed cells, UCP has been detected only in mitochondria of thermogenic adipocytes, i.e., brown and convertible adipocytes, the name UC-mitochondria was proposed for this type of mitochondria, distinguishing these organelles from common or C-mitochondria present in all other cells (Loncar and Afzelius, 1989; Loncar 1990a, 1991a).

Like other organs and tissues, the development of BAT is under strict control as regards the time and place when and where this development takes place (Wassermann, 1965; Barnard and Skala, 1970; Loncar, 1984, 1991b). The first anlage of BAT appears relatively late in the rat embryo–at 17 days of gestation–in the

Abbreviations used in this paper: BAT, brown adipose tissue; CAT, convertible adipose tissue; UCP, uncoupling protein; WAT, white adipose tissue

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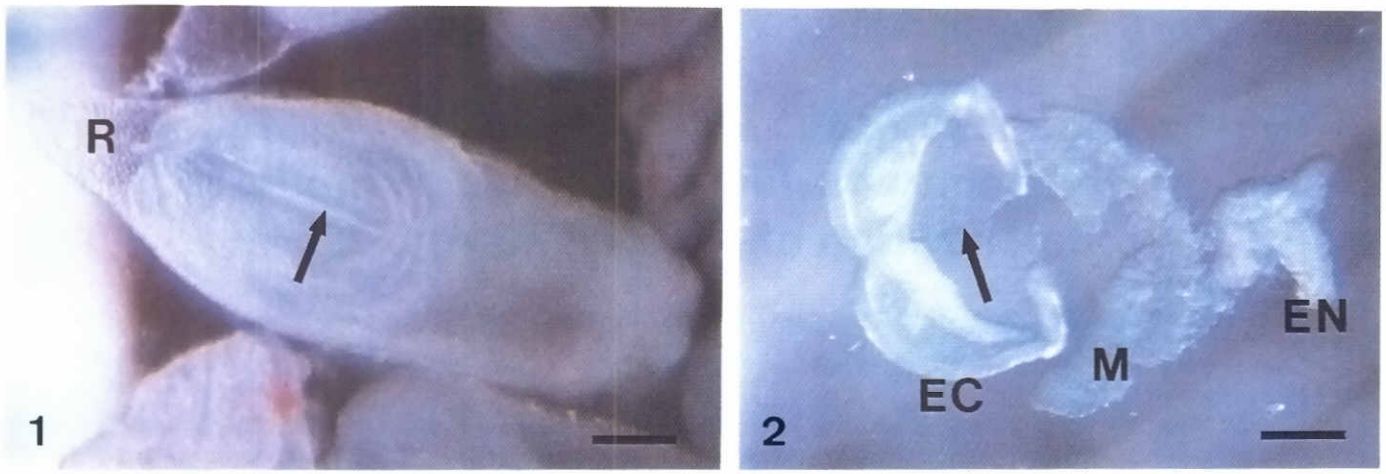


Fig. 1. Egg-cylinder of nine-day-old rat embryo. *The embryonic part occupies the anterior part of the egg-cylinder (left), where the neural groove is prominent (arrow). Reichert's membrane (R) covers the surface of the egg-cylinder. Bar: 0.2 mm.*

Fig. 2. Separated embryonic germ layers from nine-day-old rat egg-cylinder (head-fold stage). *The ectoderm (EC) is the biggest germ layer and its central part is characterized by a neural groove (arrow). The mesoderm (M) appears as a sponge-like structure in which two wings can be recognized. Immediately after separation from mesoderm, the one-cell thick endodermal germ layer (EN) has rolled up into an irregularly shaped structure. Bar: 0.2 mm.*

interscapular area, where mesenchymal cells together with penetrating blood vessels shape the lobules of BAT. After birth, in a similar way and following a general pattern of development from cranial to caudal directions, BAT appears in the thoracic area (periaortal and intercostal areas) and later in the abdominal area (Loncar, 1991b). The amount of BAT in newborn and lactating mammals constitutes maximally about 5% of the total body mass. With age this amount drops, so that BAT in mature rats at room temperature conditions amounts to 1% of total body mass (Afzelius, 1970).

Keeping in mind the above facts about the structural-functional particularity of BAT, together with the time and place of development and the amount of BAT in the life cycle of rats, it seemed peculiar that mesoderm from nine-day-old rat embryos (Figs. 1 and 2) grafted below the kidney capsule developed almost exclusively into tumors which have the appearance of BAT (Skreb *et al.*, 1976). BAT was also frequently found in tumors developed after grafting of mesoderm with ectoderm (Levak-Svajger and Svajger, 1974; Svajger and Levak-Svajger, 1974) or after grafting of mesoderm with endoderm (Svajger and Levak-Svajger, 1974).

In the present study, part of which has been presented as short abstracts (Loncar *et al.*, 1987; Loncar, 1989), mesoderm was grafted below the kidney capsule of mature male rats. One month later tumors with the appearance of BAT were analyzed. Data from structural and morphometrical analysis showed that cellularity, vascularisation and innervation of such experimentally developed adipose tissue were the same as in BAT developed in situ. These BAT-containing tumors expressed UCP mRNA, and their mitochondrial cristae contained UCP. Both injection of noradrenaline and cold stress increased the expression of UCP mRNA in the BAT-containing tumors. These stimuli also increased the amount of UCP in mitochondria of experimentally developed brown adipocytes. These data showed that although developed ectopically and in adult rats, BAT was structurally and functionally fully developed. The phenomenon, that mesoderm isolated and grafted below the kidney capsule developed almost exclusively into BAT, was explained as a result of replacing the inductive influence of ectoderm and endoderm with a new inductive influence from the neuro-vascular elements, which penetrate from the medial part of the kidney (BAT area) and induce differentiation of grafted mesoderm into BAT.

Results

Experimentally developed BAT had morphological characteristics of interscapular BAT

About 90% of mesoderm (24 tumors of 27 grafts) grafted below the kidney capsule developed into tumors. Although the size of tumors varied from a very large structure of 8x8 mm (about 15% of developed tumors) to totally resorbed tumors, the typical size of developed tumors covered a surface area of 10 to 15 mm^2. Fig. 3A and B show a typical structure of approximately 5x5x5 mm. The content of tumors also varied. 90% of tumors were made up of brown adipose tissue or a mixture of brown and white adipose tissue (Fig. 4); about 10% of tumors contained a random mixture of BAT and other mesodermal derivates: muscle, cartilage and connective tissue (Figs. 7, 8, 16 and 17). The tumors were covered by a dense fibrous capsule from which a thin layer of loose connective tissue penetrated, separating the tumor from the parenchyma of the kidney cortex (Fig. 4). The capsule usually supplied the tumors with blood vessels (Fig. 6), and some of the tumors were deeply penetrated by vessels (Fig. 4).

Brown adipocytes inside the tumors had the appearance, size and shape of brown adipocytes in the interscapular area (Figs. 4, 5 and 9) (Suter, 1969; Afzelius, 1970; Lindgren and Barnard, 1972; Loncar, 1984). In some of the tumors brown adipocytes were mixed with white adipocytes (Fig. 4), or brown adipocytes were in close contact with other mesodermal derivatives: muscle (Figs. 7 and 16), cartilage (Figs. 8 and 17) and connective tissue. These tissues were separated from brown adipocytes only by low amounts of extracellular fibers (Figs. 7 and 8). An exception was the layer of connective

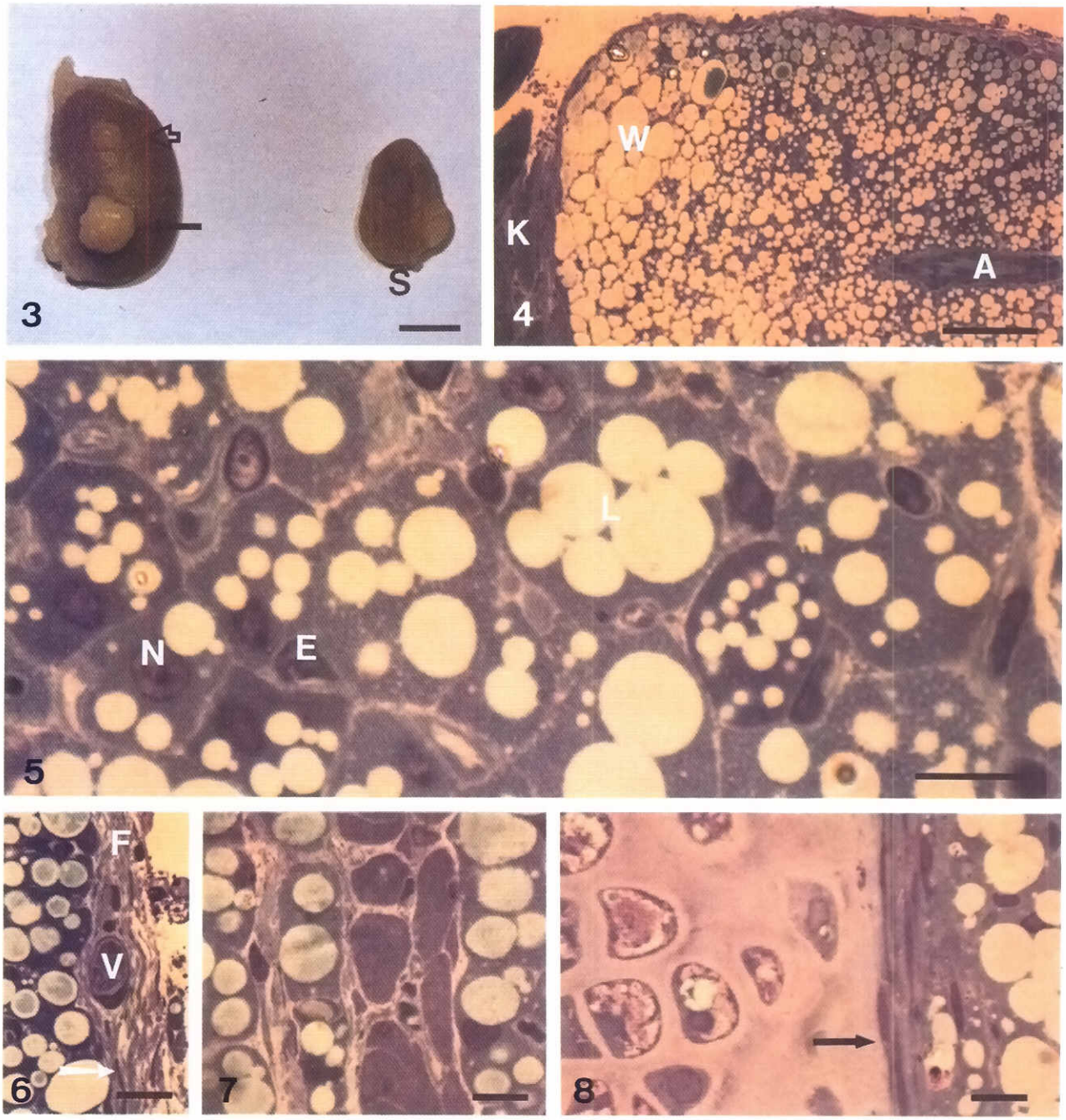


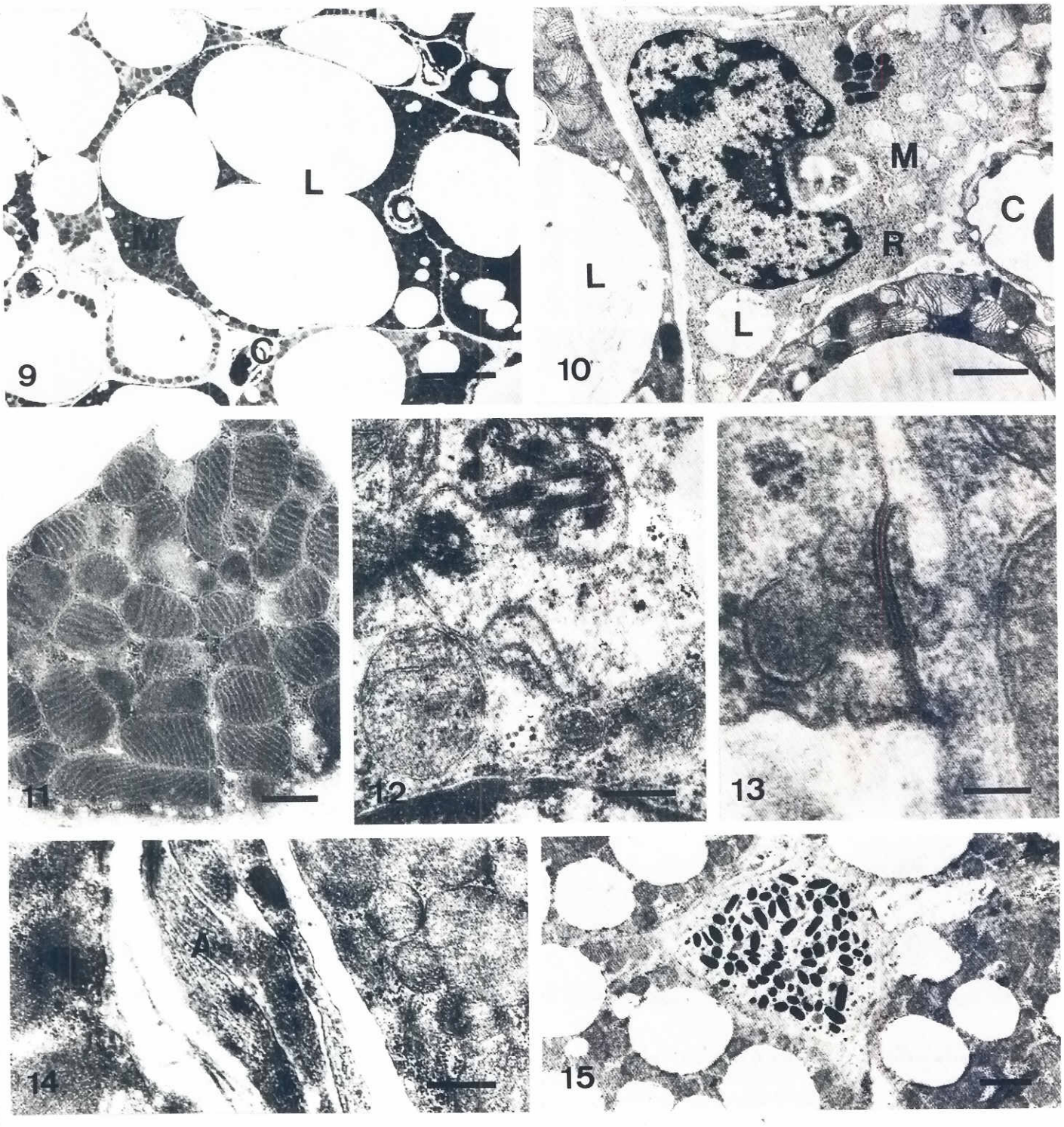
Fig. 3. Two tumors (black and open arrow) developed below the kidney capsule one month after grafting of mesoderm. *Section through the kidney (S) shows that the tumor appears as a circumscribed, well-demarcated structure which does not invade nor metastasize into kidney tissue. Bar: 10 mm.*

Fig. 4. Histological appearance of the tumor marked by a black arrow on Fig. 3. *The tumor is made up of brown adipose tissue. Some white adipocytes (W) are present at the periphery of the tumor. Note a large artery (A) situated in the center of the tumor. Kidney (K). Semithin section, toluidine blue. Bar: 40 µm.*

Fig. 5. Experimentally developed brown adipose tissue one month after grafting of mesoderm below the kidney capsule. *Brown adipocytes have nuclei (N) situated centrally, and multilocular lipid droplets (L) scattered throughout the cytoplasm. Cytoplasm of adipocytes contains crowded mitochondria (small dark blue dots inside all adipocytes). Nucleus of endothelial cells (E). Semithin section, toluidine blue. Bar: 10 µm.*

Fig. 6. The surface of the tumor is covered by a fibrous capsule (F) which contains large blood vessels (V). *Fibrocyte (arrow). Semithin section, toluidine blue. Bar: 10 µm.*

Figs. 7-8. In two mesodermal tumors, other mesodermal products like muscle (7) or cartilage (8) are present between brown adipocytes. *Thin perichondrium (arrow). Semithin section, toluidine blue. Bar: 10 µm.*



Figs. 9-15. Electron micrographs of BAT developed one month after mesoderm grafting below the kidney capsule. (9) *Cytoplasm of brown adipocytes with crowded mitochondria (M). Numerous lipid droplets (L) are scattered throughout the cytoplasm. The surface of each adipocyte is in close contact with several capillaries (C). Bar: 2 μm.* **(10)** *Preadipocyte situated between mature adipocytes. The ground cytoplasm of the preadipocyte contains numerous ribosomes (R). Mitochondria are in different stages of development (M). Lipid droplet (L), capillaries (C). Bar 0.5 μm.* **(11)** *Ellipsoidal mitochondria of brown adipocyte with numerous cristae occupy most of the cytoplasm. Bar: 0.3 μm.* **(12)** *Irregular mitochondria with different inclusions and irregular cristae are present in some experimentally developed brown adipocytes. Bar: 0.25 μm.* **(13)** *One of the numerous gap-junctions between brown adipocytes. Bar: 0.20 μm.* **(14)** *A bundle of axons (A) between brown adipocytes. Bar: 0.3 μm.* **(15)** *Mast cell situated between brown adipocytes. Bar: 1 μm.*

tissue about 30 μm thick which surrounded cartilage and which resembled perichondrium. Inside the perichondrium, chondrocytes in different stages of development were located (Figs. 8 and 17).

The ultrastructural characteristics of the brown adipocytes (Fig. 9) in tumors were essentially the same as those of interscapular brown adipocytes, as described earlier (Suter, 1969; Afzelius, 1970; Lindgren and Barnard, 1972, Loncar *et al.*, 1988b, Loncar, 1990a, 1991b). Morphometrical analysis showed that the mean maximal diameter of brown adipocytes was about 25 μm (Table 1). Lipids were dispersed through the cytoplasm of brown adipocytes in the form of small droplets and occupied about 60% of adipocyte volume (Table 1). The size, shape and amount of mitochondria per cytoplasm was similar to those in adipocytes of 3-4 week old rats (Lindgren and Barnard, 1972). Besides normally developed mitochondria (Fig. 11), some mitochondria in brown adipocytes of BAT-containing tumors contained different inclusions (Fig. 12).

In one-month-old tumors, preadipocytes at different levels of maturation were also present between fully developed brown adipocytes (Fig. 10). These preadipocytes were in close contact with mature brown adipocytes which were interconnected with other brown adipocytes by numerous gap-junctions (Fig. 13). Numerous capillaries ran between adipocytes covering about one-third of the adipocyte surface (Figs. 5 and 9; Table 1). The rest of the brown adipocyte surface was covered with the fibers of extracellular matrix, which appeared as collagen fibers (not shown). Axons of numerous nerves ran close to capillaries as well as through the narrow intercellular space (Fig. 14).

Morphological analysis showed that other cells besides brown adipocytes were present in two tumors: for example, mast cells (Fig. 15), fibrocytes (Fig. 16), white adipocytes (Fig. 4), muscle fibers (Figs. 7 and 16), and chondrocytes (Fig. 8 and 17). All these cells had an ordinary appearance as described elsewhere (Weiss, 1983; Krstic, 1984). An exception was peculiar cells that developed in one tumor. Between the fibers of skeletal muscle or between these fibers and brown adipocytes, cells with very well developed mitochondria were present (Fig. 16). In the cytoplasm of these cells, instead of lipid droplets, numerous unorganized myofilaments were present, so that the cells had the morphological characteristics both of brown adipocytes and of myoblasts (Fig. 16).

Experimentally developed BAT contained UCP mRNA and UCP

The positive signal after the slot blot determination of UCP mRNA in tumors containing BAT showed that the experimentally developed BAT had the ability to express brown adipocyte-specific mRNA. As Fig. 20 shows, the amount of UCP mRNA expressed in four different tumors differed. However, immuno-electron microscopic determination of UCP showed that mitochondria of brown adipocytes contained the UCP distributed over mitochondrial cristae (Figs. 18-19) similar to the mitochondria of brown adipocytes of several other species (see Loncar, 1990a,b, 1991a,b).

Noradrenaline injections and cold stress increased the expression of UCP mRNA and UCP in experimentally developed BAT

Cold stress caused BAT in tumors to appear in the same way as described earlier for interscapular BAT (Suter, 1969; Lindgren and Barnard, 1972; Loncar *et al.*, 1988b). Immuno-electron microscopical determination of UCP showed that in experimentally developed BAT the amount of UCP per mitochondrial cristae was higher than in the host's interscapular BAT, though the difference was not significant (Fig. 21). Noradrenaline (Fig. 21) and cold stress (Figs. 19 and 21) increased the amount of UCP in experimentally developed BAT. As Fig. 20 shows, cold stress also significantly increased the amount of UCP mRNA in the experimentally developed BAT as well as in the interscapular BAT.

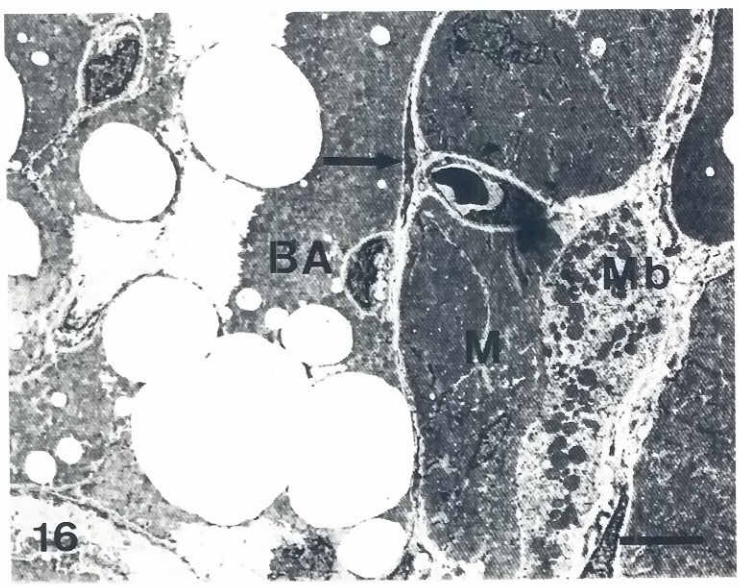
Discussion

Experimentally developed BAT

Although developed experimentally in an atypical place and at an atypical time, the BAT in all analyzed tumors had the characteristics of typical, ordinary BAT. In this way the results presented here confirm earlier histological description of tumors that contained BAT (Levak-Svajger and Svajger, 1974; Svajger and Levak-Svajger, 1974; Skreb *et al.*, 1976). It is remarkable that in all analyzed tumors the ultrastructural order (but not the histological one) was almost perfect, i.e., each type of cells had specific and typical organelles. The only exception was the appearance of cells with developed mitochondria and irregular myofilaments in some tumors (Fig. 16). Without further studies it is not possible to determine whether these cells should be classified as myoblasts or as «hybrid-cells» which express the morphological characteristics of both brown adipocytes (mitochondria) and myoblasts (myofilaments).

Mitochondria of the brown adipocytes in tumors were regularly developed, and immuno-electron microscopical analysis revealed that they contained UCP. Distribution of the UCP in mitochondrial cristae and the amount of UCP per mitochondria were slightly higher in experimentally developed brown adipocytes than in interscapular brown adipocytes of the host. The structural-functional characteristics of BAT in rats partially depend on their age and on the ambient temperature. Previous results concerning BAT of newborn rodents (Suter, 1969; Lindgren and Barnard, 1972; Loncar 1984) showed that BAT increased after birth. During that time new brown adipocytes arise and their cytoplasm accumulates lipids and specific UC-mitochondria. If rats are raised in room temperature conditions the amount of the brown adipocyte mitochondria (expressed as mitochondrial cristae per brown adipocyte cytoplasm) reaches a peak at about 2-3 weeks after delivery (Lindgren and Barnard, 1972). After this period the amount of mitochondria (cristae) declines, and the mitochondrial content of older rats is about 70% of the mitochondrial content of brown adipocytes in young rats.

The results described here showed that BAT in tumors, developed below the kidney capsule, had the structural characteristics of interscapular BAT of about 3-week-old rats (Suter, 1969; Lindgren and Barnard, 1972). The amount and distribution of gold particles, revealing the presence of UCP, showed that the amount of UCP in brown adipocytes of experimentally developed tumors was similar to the amount of UCP in interscapular brown adipocytes of 3-week-old rats (Loncar, unpublished). Data about determination of UCP mRNA had only qualitative meaning. Structural changes and changes in the amount of UCP (Figs. 19 and 21, Table 1), as well as the results showing increased amounts of UCP mRNA (Fig. 20), showed that in BAT-containing tumors both noradrenaline and cold stress induced changes similar to those described for interscapular BAT (Suter, 1969; Ricquier *et al.*, 1983; Peachey *et al.*, 1988; Silva, 1988; Geloen and Trayhurn, 1990; Herron *et al.*, 1990). Also using immuno-electron microscopy as a quantitative method (Slot *et al.*, 1989) here it is shown clearly that in tumors of cold-stressed animals the amount of UCP per μm^2 of mitochondrial cristae was about 2.5 fold higher than in tumors in room-acclimated rats. These data corroborate data about the direct *in vivo* measurement of UCP where it has shown even more than a 3-fold increase of UCP after cold stress (Rafael *et al.*, 1985; Trayhurn *et al.*, 1987). Thus, in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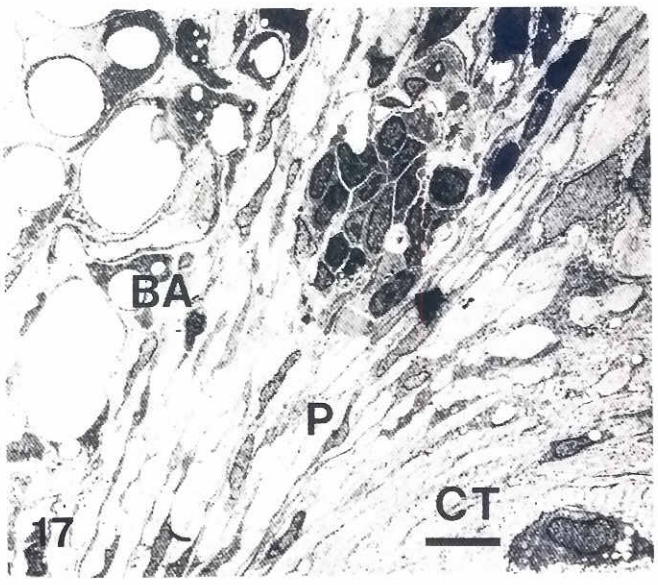
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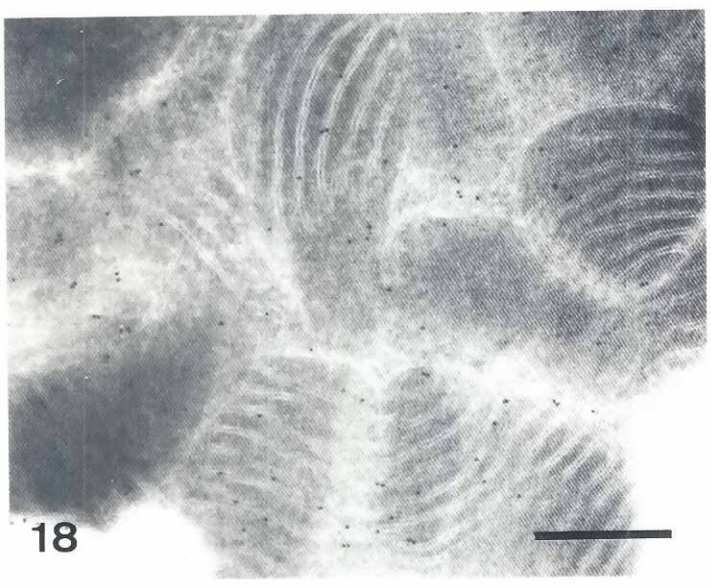
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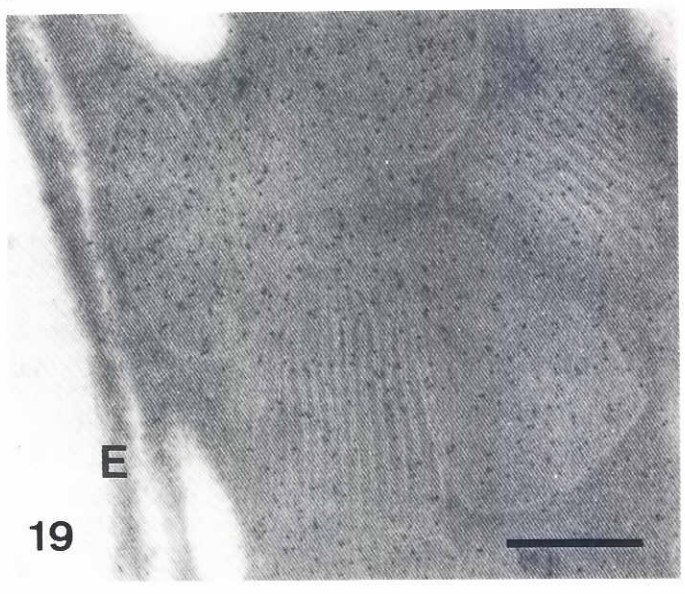
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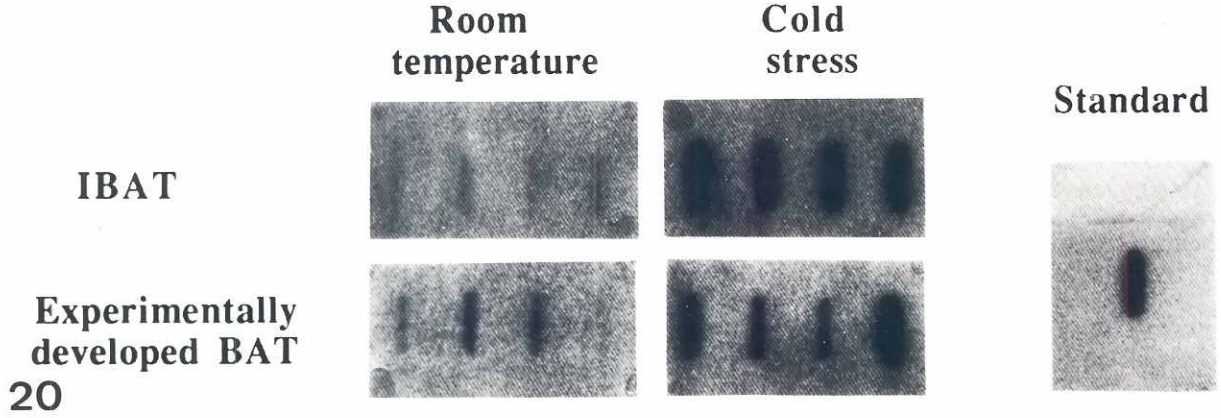
Room temperature
Cold stress
Standard
IBAT
Experimentally developed BAT
20

addition to structural characteristics, experimentally developed BAT displayed physiological thermogenic features (expressed as the amount of UCP) in the same way as interscapular BAT (Loncar 1990a,b, 1991b)

Svajger *et al.* (1981) have reported that ectoderm grafted below the kidney capsule undergoes adaptive changes for a couple of days. It could be anticipated that mesoderm probably needs a similar period of adaptation. If mesoderm needs a similar period for adaptation, this means that tissues in one-month-old tumors would appear younger (as about 3 weeks old). The fact that BAT in one-month-old tumors had the structure of interscapular BAT of 3-week-old rats confirms these expectations, and indicates that, once established, anlage for BAT follows the developmental pattern as strictly as if it had been developed *in situ*. These experimentally developed brown adipocytes contained specific UC-mitochondria, although it is clear that rats weighing about 400 g or more and kept in room temperature conditions (20±2°C) do not need these specific UC-mitochondria for nonshivering thermogenesis. Thus, both brown adipocytes and UC-mitochondria were developed as specific items and, once established, anlage for BAT will develop in this tissue regardless of the thermogenic needs of animals.

The results concerning experimentally developed BAT also strongly support the idea of the existence of another type of thermogenic adipose tissue, described as convertible adipose tissue (CAT) (Loncar and Afzelius, 1989; Loncar, 1991a,b). In so-called bigger mammals (cat, rabbit, cow) CAT exists during the perinatal period in certain areas as thermogenic adipose tissue that was until recently considered as BAT. However, with growth, there is no need for UC-dependent thermogenesis, and CAT becomes like WAT (adipocytes accumulate lipids, UC-mitochondria are destroyed and replaced with C-mitochondria). In small rodents and hibernators, BAT has multilocular brown adipocytes containing UC-mitochondria even in maturity (Loncar, 1990a). The fact that grafted mesoderm in mature rats mostly developed into BAT indicates that the appearance and development of a particular type of adipose tissue depends on developmental mechanisms, not on temporary thermogenic or metabolic requirements. Thus, the appearance and distribution of BAT or CAT in animals depends on the type of anlage which develops into a particular type of tissue.

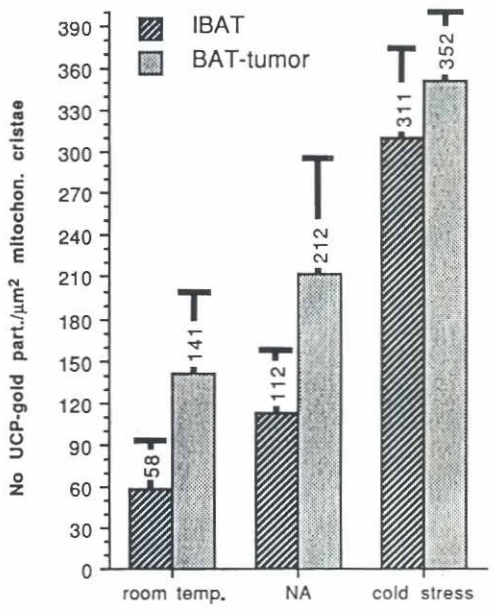

Fig. 21. The amount of UCP (measured as the number of UCP-gold particles per μm^2 mitochondrial cristae) in experimentally developed BAT in rats exposed to different temperatures or in rats injected with noradrenaline. *One month after grafting of mesoderm below the kidney capsule, rats bearing BAT-tumors were separated into three groups (three rats per group). The first group remained caged at room temperature (20± 2°C). The second group of rats caged at room temperature was injected with noradrenaline (NA) (5 μmol. noradrenaline/kg body weight per day) for 4 days. The third group of rats was exposed to cold stress (+4°C and -20°C) for one week. Numbers of UCP-gold particles (± S.D.) per μm^2 of mitochondrial cristae are UCP-gold particles (subtracted for background labeling) labeled over mitochondrial cristae. Mitochondria of experimentally developed brown adipocytes contain more UCP than mitochondria of interscapular brown adipocytes which serve as a positive control. The injection of NA increased (but not significantly) the amount of UCP. Significant increase of UCP per mitochondrial cristae is recorded in mitochondria of brown adipocytes of cold-exposed rats.*

Why BAT?

What are the developmental mechanisms that direct mesoderm from a rat embryo nine days old to develop so frequently into BAT? One can extend these questions by considering that in a large series of about 150 grafts, where mesoderm and mesenchyme were grafted below the kidney capsule, these developed almost exclusively into BAT (Skreb *et al.*, 1976). Comparing the development of mesoderm *in situ* with the development of grafted mesoderm,

Figs. 16-17. The atypical «neighborhood» developed in some tumors after mesoderm grafting. (16) *Brown adipocytes (BA) are in tight contact with striated muscle (M). Note the cell (Mb) with well developed mitochondria and irregular myofilaments. Fibrocyte (arrow). Bar: 2 μm.* **(17)** *Only a thin perichondrial layer (P) separates brown adipocytes (BA) from cartilage tissue (CT). Bar: 4 μm.*

Figs. 18-19. Immuno-electron micrographs of experimentally developed BAT after mesoderm grafting below the kidney capsule. The distribution of gold particles indicates the presence of uncoupling protein (UCP). (18) *Mitochondrial cristae of brown adipocytes of room-acclimated rat have a small amount of UCP. Bar: 0.25 μm.* **(19)** *The cold-stressed rats have mitochondrial cristae containing numerous UCP molecules. The ground cytoplasm of brown adipocytes and the cytoplasm of adjacent endothelial cells (E) contain sporadic gold particles, which represent background labeling. Bar: 0.25 μm.*

Fig. 20. The expression of UCP mRNA in experimentally developed BAT. *One-month-old tumors were homogenized and their mRNA was isolated, blotted, and hybridized with P^{32}-labeled cDNA probe for UCP mRNA. Dark lines and the level of darkness on the autoradiograph (compared with standard) demonstrate the presence and the amount of UCP mRNA in interscapular BAT (IBAT) and in experimentally developed BAT. Each line represents UCP mRNA pooled from one tumor or from interscapular BAT of one rat. Standard represents UCP mRNA pooled from interscapular brown adipose tissue of four mice exposed to +4°C for 48 h. The tissue in the tumors expressed UCP mRNA (see Experimentally developed BAT- Room temperature). Cold stress increased the expression of UCP mRNA in the experimentally developed BAT in the same way as in the interscapular BAT. The amount of UCP mRNA in tumors varies (different intensity of darkness), depending on the amount of BAT in tumors.*

TABLE 1

MORPHOMETRIC PARAMETERS (±S.D.) OF EXPERIMENTALLY DEVELOPED BROWN ADIPOSE TISSUE (BAT) AND INTERSCAPULAR BROWN ADIPOSE TISSUE (IBAT)

	BAT developed experimentally	IBAT developed *in situ* (control)
Mean maximal diameter of adipocytes (μm)	23.5±1.9	43.1±8.3
Number of capillaries per adipocyte (n)	3.3±0.6	3.0±0.4
Surface of adipocyte covered by capillaries (%)	34±11	29±7
Adipocyte volume occupied by lipid droplets (%)	59±17	67±13
Cytoplasm of adipocytes occupied by mitochondria (%)	64±7	58±9
Surface of mitochon. cristae per mitochon. ($\mu m^2/\mu m^3$)	41.8±5.5	35.5±6.1
Surface of mitochon. cristae per cytoplasm ($\mu m^2/\mu m^3$)	27.7±3.3	20.5±5.2
Surface of mitochon. cristae per adipocyte (mm^2/adipocyte)@	0.07	0.28
Number of UCP-gold particles per cristae ($N/\mu m^2$)	141±49	58±37
Number of UCP-gold particles per mitochondria ($N \times 10^3/\mu m^3$)	5.8±2.0	2.0±1.3
Number of UCP-gold particles per cytoplasm ($N \times 10^3/\mu m^3$)	3.9±1.3	1.1±0.7
Number of UCP-gold particles per adipocyte ($N \times 10^6$)@	10.8	15.2

@ Recalculated data from above measurement

some paradoxes have appeared: a) In grafting experiments (with various combinations of germ layers) the incidence of tumors which contained BAT was very high (Levak-Svajger and Svajger, 1974; Svajger and Levak-Svajger, 1974; Skreb *et al.*, 1976), although BAT is one of the last tissues to appear during normal development; the first traces of BAT appear in interscapular areas of the rat embryo between 17 and 18 days of gestation (Barnard and Skala, 1970; Loncar 1984). b) WAT is another type of tissue that is relatively frequent in tumors (in combination with BAT or alone), and yet the first white preadipocytes appear in the rat embryo even later than BAT; the first anlage of WAT appears in the inguinal area between the 19th and the 20th day of gestation of the rat (Loncar, unpublished). c) Cardiac myoblasts appear as early as in the 10th day of gestation in the rat embryo, but the specific cardiac muscle was absent in tumors originating from grafted mesoderm. It was not possible, even under EM, to distinguish the type of striated muscle (cardiac/skeletal) developed in one tumor.

The above data about the histological composition and the content of grafted mesodermal tumors indicate that the differentiation of 9-day-old mesoderm grafted below the kidney capsule does not follow the pattern of differentiation which occurs during differentiation *in situ*. The development and differentiation of the mesoderm *in situ* is under the inductive influence of ectoderm and endoderm (Maclean and Hall, 1987; Sharpe and Ferguson, 1988; Gilbert, 1991). However, extirpated from this natural inductive environment, 9-day-old mesodermal cells displayed limited developmental potential. This phenomenon was also confirmed by *in vitro* experiments. Isolated mesoderm grown in culture conditions failed to develop into any specified histological structures or cells (Loncar, in preparation). As a significant difference, isolated mesoderm from 10-day-old rat embryo differentiates into a variety of mesodermal products, including skeletal and cardiac muscle (Svajger and Levak-Svajger 1976).

Experiments with transplantation of adipose tissue, either BAT or WAT, have shown that the new local environmental conditions have stronger influences on the future of the grafts than the grafts' genetic pedigree (Ashwell, 1985; Ashwell *et al.*, 1986). These data together with the fact that products of mesenchymal cells (fibroblasts, chondroblasts, osteoblasts, myoblasts) have the ability to interchange from one cell type to another (Gabbiani and Rungger-Brandle, 1981; Marziasz and Jones, 1990), could explain why mesodermal cells in the environment below the kidney capsule can develop into BAT so frequently. Embryonic germ layers (mesoderm) grafted below the kidney capsule can survive only if the tissue becomes vascularized from the surrounding area; i.e., from the capsule or from the kidney cortex. The hilus of the kidney is surrounded by BAT, and the penetration of blood vessels with adrenergic nerves (Simon, 1965; Barnard *et al.*, 1980) from the hilus probably initiates the differentiation of grafted mesodermal cells into BAT (Fig. 22). Alternatively, if vessels penetrate the mesoderm-grafting area from the lateral side of the kidney (this part of the kidney is covered with WAT), they could facilitate the development of WAT (Fig. 22). The penetration of neurovascular elements from both sides would result in the development of WAT-BAT mixed tumors.

Two tumors in addition to brown adipocytes contained other mesodermal products (islands of cartilage, muscle and connective tissue). This indicates that factors such as the amount and the exact developmental stage of grafting mesoderm, «contamination» of grafted mesoderm with endodermal or ectodermal cells, the amount and the type of surviving grafted mesodermal cells, etc., could influence the process of differentiation of grafted mesoderm. Further studies are needed to explain the influence of these different parameters on the development and differentiation of isolated mesoderm.

Materials and Methods

Animals and isolation of mesoderm

The basic technique for the isolation of mesoderm is described in detail elsewhere (Levak-Svajger *et al.*, 1969; Svajger and Levak-Svajger, 1975); I have used this technique with some modifications. Sprague-Dowley rats, whose gestation was considered to have begun early in the morning of the day when sperm was found in the vaginal smear, were sacrificed (after deep anaesthesia with Ketalar) on the 9th day of gestation. The egg cylinders were isolated (Fig. 1). After removing the Reichert's membrane and the extraembryonic parts, embryonic shields were treated with a mixture of 1% trypsin (Sigma) and 5% pancreatin in Dulbecco's Modification of Eagle's

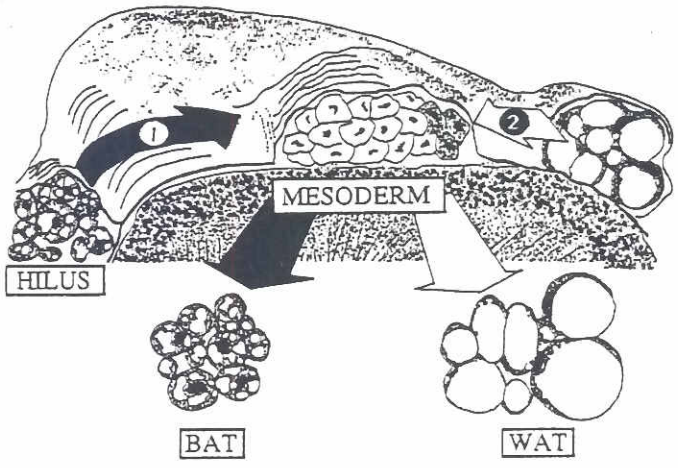

Fig. 22. Schematic drawing of the differentiation of 9-day-old mesoderm isolated and grafted below the kidney capsule. *In the new environment, mesodermal cells are devoid of instructive influence of ectoderm and endoderm. The penetrating neuro-vascular elements (1) from the hilus of the kidney (BAT area) brings inductive signal(s) facilitating the differentiation of mesodermal cells into BAT. If neuro-vascular elements penetrate the grafted area from the lateral side (2) of kidney (WAT area), the cells of the grafted mesoderm differentiate into WAT. Mixed tumors (which contain BAT and WAT) develop when mesoderm is penetrated by neuro-vascular elements from both sides (1+2) of the kidney.*

medium (Flow Laboratories). The enzymatic reactions were stopped with bovine serum 45 min later. During this treatment the ectoderm was detached from the underlying mesoderm, spontaneously or with the help of tungsten needles. Needles were also used to separate the mesoderm from the endoderm. Mesoderm from 9-day-old rat embryo is a homogeneous mass of cells that does not yet show any specific, spatial (cardiac/somitic) differences. (Fig. 2). Mesoderm was transferred by means of a braking pipette under the capsule of the left kidney of adult male rats (~400 g). One month later the recipients were separated into three different groups.

One group of recipients was kept at room temperature conditions (20°±2°C). Another group of recipients was transferred to a cold room (+4°C) for one week. During that time these rats were exposed to cold stress (-20°C) two times daily for one h (Loncar *et al.*, 1988a). A third group of rats was kept at room temperature and injected with noradrenaline for 4 days (5 µmol. of arterenol bitartarate (Sigma) per kg of body weight per day). The interscapular BAT of recipients served as a positive control.

Electron microscopy and morphometry

Animals were anesthetized and were transcardially perfused first with Ringer solution (ml/g body weight, warmed to 37°C, containing 10,000 U of heparin per 1 l of Ringer) and then with a fixative. The fixative used was 2% glutaraldehyde and 1% paraformaldelhyde in 0.1M phosphate buffer which contained 0.1M sucrose. Grafts which had developed below the kidney capsule were either excised *in toto* or microdissected in pieces 1 mm^3. They were immersed in the fixative (see above) for 24 h, then rinsed in 0.1M phosphate buffer, postfixed in 1% OsO_4, dehydrated and embedded in Epon. All tumors were serially sectioned and prepared for electron microscopy analysis as described earlier (Loncar *et al.*, 1986).

Morphometrical measurements as described in detail elsewhere (Loncar *et al.*, 1986) were done on the following parameters:

a) the mean maximal diameter of adipocytes;
b) the number of capillaries per adipocyte;
c) the adipocyte surface covered by capillaries;
d) the volume of adipocytes occupied by lipids;
e) the amount of adipocyte cytoplasm occupied by mitochondria;
f) the surface of mitochondrial cristae per 1 μm^3 of mitochondria;
g) the surface of mitochondrial cristae per 1 μm^3 of cytoplasm;
h) the surface of mitochondrial cristae per adipocyte;
i) the number of UCP-gold particles per 1 μm^2 of cristae;
j) the number of UCP-gold particles per 1 μm^3 of mitochondria;
k) the number of UCP-gold particles per 1 μm^3 of adipocyte cytoplasm;
l) the number of UCP-gold particles per adipocyte;

Parameters *a-c* were determined on semithin sections. Parameters *d-h* and *i-l* were determined on ultrathin plastic and ultrathin cryo sections respectively.

Determination of UCP mRNA

Tumors from room-temperature or cold-acclimated animals (injected and noninjected) were homogenized in guanidine extraction buffer. Total RNA was isolated according to Jacobsson *et al.* (1985). For the slot blots, an amount of the RNA preparation corresponding to 4 µg RNA was dissolved in 300 µl 10 x SCC/18% formaldehyde and diluted with H_2O to yield a total of 400 µl. After incubation for 15 min at 65°C, this solution was applied to a Zetaprobe filter in a Minifold slot-blot apparatus, washed with 400 µl 10xSCC and dried at room temperature. After prehybridization with salmon sperm DNA (Sigma) and poly A/poly C mixture (Jacobsson *et al.*, 1985), the filter paper was hybridized with cDNA probes, then nick translated with a Bethesda Research Laboratories kit. The uncoupling protein probe utilized was the one characterized earlier by Jacobsson *et al.* (1985).

Determination of UCP by immuno-electron microscopy

Polyclonal rat anti-UCP-antibody was prepared as described by Cannon *et al.* (1982). Specificity of UCP labeling was tested as described earlier (Loncar, 1990a). For immuno-electron microscopical analysis of UCP, tumor-bearing rats were fixed as described above. Small pieces (0.5-1 mm^3) of tumors, having undergone overnight fixation, were washed in phosphate buffer and transferred to polyvinyl pyrrholidon; two hours later, this tissue was transferred to silver pins and frozen in liquid nitrogen. The preparation of grids, cutting of frozen tissue, labeling with anti-UCP antibody, decorating with protein-A-gold complex, contrasting of sections with uranylacetate oxalate, and embedding of grids in methyl cellulose was done as described earlier (Loncar, 1990a).

Acknowledgments

The author gratefully acknowledges technical support and stimulating discussion from the laboratories of Anton Svajger, Barbara Cannon and Björn A. Afzelius. The author is thankful to Björn A. Afzelius and Anton Svajger for their valuable comments and criticism on this manuscript. This study was supported by grants from the Swedish Natural Science Research Council and partially by a grant from the Scientific Fund of the Republic of Croatia, Zagreb.

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Accepted for publication: April 1992