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IN VITRO INDUCTION OF THE FAT-STORING PHENOTYPE IN A LIVER CONNECTIVE TISSUE CELL LINE-GRX

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SUMMARY

Liver connective tissue cells have been characterized as perisinusoidal myofibroblasts and hepatic lipocytes (Ito cells, fat-storing cells). A concept of a single mesenchymal cell population that may be modulated between these two phenotypes has been postulated. We have previously established a continuous murine cell line, GRX, obtained from fibrotic granulomatous lesions induced by schistosomal infection in mouse liver. This cell line is considered to represent liver myofibroblasts. In the present study we have induced the conversion of these cells into lipocyte (fat storing) phenotype by treatment with insulin and indomethacin. We have quantified the lipid synthesis and the increase of activity of involved enzymes during the induction of the fat-storing phenotype and described modifications of cell organization along this modulation of cell functions.

Key words: liver; lipocyte; Ito cell; myofibroblast; perisinusoidal cell; insulin; indomethacin.

INTRODUCTION

In normal liver, the ratio between connective tissue components and hepatocytes is particularly low. However, liver injuries may induce a prompt response of mesenchymal cells. Depending on the nature of the injurious agent, its intensity, and its duration, an extensive liver fibrosis may develop, with great increase of both connective tissue cells and extracellular matrix. Increase of liver connective tissue cell populations is thus associated and probably causally related with the establishment of hepatic fibrosis.

Liver connective tissue cells have been extensively studied in recent research. In vitro analysis of connective tissue cells isolated from fibrotic livers of humans and experimental animals has shown that they belong to the smooth muscle cell lineage and may be characterized as myofibroblasts (5,23,29,30,35,40). Their counterpart in vivo may be the perisinusoidal cells, characterized also as myofibroblasts (4). Similar cells were identified in hepatic tissues attained by fibrosis (3,16,31,34). On the other hand, liver perisinusoidal fat-storing cells (lipocytes or Ito cells) have also been shown to be reactive in various liver injuries, and their transformation into myofibroblasts has been documented (24,28). A concept of a single hepatic mesenchymal cell population that may be modulated between myofibroblast and lipocyte phenotypes has been postulated (4,24).

Hepatic lipocytes have been obtained from rat livers and maintained in primary cultures (22). They have been shown to divide and actively secrete a complex extracellular matrix (10,14,21,43). Along their in vitro cultivation, lipocytes decrease their lipid content and acquire a more fibroblastoid phenotype (11,43). It is not known if this change represents a general de-differentiation, observed in many mesenchymal cells that acquire fibroblastoid morphology in vitro, or a shift between the two phenotypes of liver perisinusoidal cells. The corresponding changes required for conversion of the myofibroblast phenotype into the fat-storing one have not been characterized.

We have established in vitro a continuous cell line named GRX, isolated from fibrotic granulomas induced in mice livers by experimental infection with *Schistosoma mansoni* (6). We consider that this cell line represents murine LCTC. A fraction of these cells accumulate in in vitro lipids, and clones have been isolated with increased ability to be converted into fat-storing cells.

We have shown that, similar to the in vivo induction of Ito-cell hyperplasia by retinoids, the GRX cell line may be induced in vitro to convert into lipocytes by retinol (25). However, beyond their essential function in the retinol metabolism, liver perisinusoidal cells have apparently a complex role in the maintenance of liver architecture and normal or pathologic secretion of extracellular matrix (4,8,26). These activities are under the control of general mediators of tissue homeostasis. By analogy with the induction of adipocyte differentiation, we have tested now the activity of insulin on the induction of the lipocyte phenotype. Insulin alone, in concentration used in classical works on adipocyte differentiation

(1,12,15) have a slow but discernible inducing activity on GRX cells after a period of incubation superior to 14 d. Although this is similar to adipose differentiation, in our model this inducing time was too long: in contrast to adipocytes that are contact-inhibited, GRX cells overgrow and form irregular cultures that eventually contract into spheroids and detach from the culture vessel. Similar to results described by Williams and Polakis (41) and Verrando et al. (39) for adipocyte differentiation, we have observed that indomethacin had a strong effect on the fat storing in GRX cells, inducing the lipocyte phenotype in 4 to 5 d.

The present study was done to quantify the lipid synthesis and the increase of activity of the involved enzymes during the induction of fat-storing phenotype and to describe modifications of cell organization along this modulation of cell functions.

Materials and Methods

Cell cultures. GRX-123 cells were routinely maintained in Dulbecco's minimum essential medium (DMEM) (Sigma Chemical Co. St. Louis, MO) with 10% fetal bovine serum (FBS) (Cultilab, Campinas, SP, Brazil) and 3 g/liter HEPES buffer (Sigma), under normal air atmosphere.

We have monitored cell proliferation in cultures plated at the density of 5×10^5 cells/25-cm² tissue culture flasks. After 24 h we have added insulin or indomethacin or both, and quantified cells by direct counting after 2, 5, 8, and 12 d.

Induction of the fat-storing phenotype. Routinely, the fat-storing phenotype was induced by incubation of cultures with DMEM supplemented with 5% FBS, 0.13 m*M* indomethacin and 0.17 μM insulin (Sigma). Lipid inclusions were identified after staining with the saturated Oil Red O (Sigma) solution in 70% ethanol.

Total lipid quantification was done by the sulfophospho-vanilin colorimetric method using the commercial kit Merckotest 3321 (Merck S. A., Rio de Janeiro, Brazil), following the manufacturer's instructions. Lipids were expressed in function of total protein, determined by the Coomassie brilliant blue G250 colorimetric method (36), or in function of total DNA, determined by the method of Burton (7).

Lipid synthesis was estimated by incorporation of 2-[^{14}C]sodium acetate (51.0 mCi/mmol, New England Nuclear, Boston, MA). Cells were incubated for 24 h in the presence of radiolabeled acetate (1 μCi/25-cm² tissue culture flask containing 5 ml of medium). Total lipids were extracted with chloroform:methanol (2:1) and the radioactivity was quantified by a Beckman L803 liquid scintillator.

Cytochemistry. For detection of intracellular glycerol-3-phosphate dehydrogenase (GPDH), we have adapted techniques described by Rieder et al. (33) and Hausman et al. (18). Cells were washed with balanced salt solution (BSS) at 37° C for 5 min. Lipids were extracted by 2.5-min immersion in iced acetone, 5-min immersion in chloroform (−20° C), and 1-min immersion in iced acetone. They were incubated with BSS containing 0.01% Triton X-100, buffered at pH 8.4 with 10 m*M* tris at 37° C for 5 min. They were subsequently incubated 15 min in the same buffer containing 0.3 mg/ml Thiazolyl blue (MTT), 0.15 mg/ml of phenazin methosulfate, 0.5 m*M* nicotinamide adenine dinucleotide (NAD), 10 m*M* hydrazine, and 10 m*M* racemic glycerol-3-phosphate (all from Sigma). In control experiments, glycerol-3-phosphate was omitted or substituted by 10 m*M* lactate (Sigma).

Intracellular esterase activity was demonstrated with naphtol AS-D-chloroacetate as substrate, using the Sigma research kit (no. 90) following the manufacturer's instructions.

Electron microscopy. Cell cultures were fixed following the method described by Hirsch and Fedorko (19). Briefly, cells were washed with BSS and fixed in a mixture of glutardialdehyde and OsO_4 solutions in cacodylate buffer for 30 min at 4° C. They were rinsed and treated with uranyl acetate at pH 6.3 for 30 min. Cultures were subsequently dehydrated in ethanol and embedded in epoxy resin. Ultrathin sections were stained by standard uranyl acetate and lead citrate solutions and observed under a Phillips 300 electron microscope.

Results

When maintained in standard culture conditions, GRX cells were stellate. They changed into elongate cells organized in strands and whirls after confluence. Similar to other cells of the smooth muscle lineage, GRX cells had low contact inhibition and overgrew into typical "hills and valleys."

The growth curve for GRX cells, maintained under standard conditions and in the presence of insulin, indomethacin, or both, are presented in Fig. 1. The cell

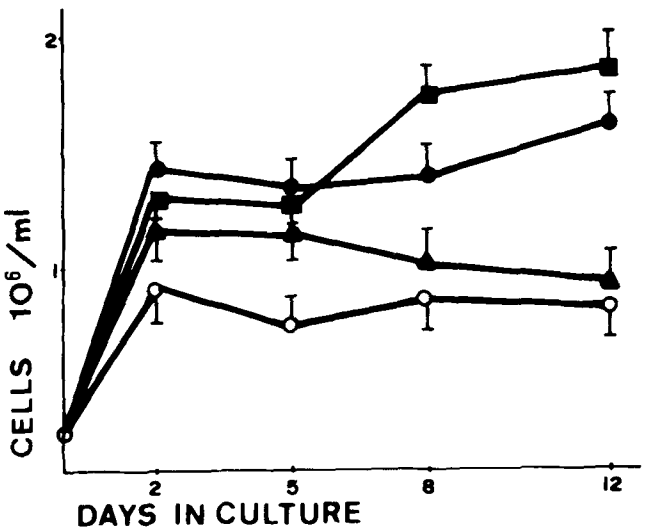

FIG. 1. Proliferation of GRX-123 cells in DMEM containing 5% FBS (*solid circles*), supplemented with 0.17 μM insulin (*solid squares*), 0.13 m*M* indomethacin (*open circles*), and with both drugs (*solid triangles*). Cells were harvested in 2 ml of BSS and counted. Mean values and SD for three independent experiments are shown.

number observed in cultures treated with insulin was slightly increased as compared to controls. In the presence of indomethacin, with or without insulin, cultures attained the stationary phase of growth after 2 d. This cytostatic activity was not due to the cytotoxicity of indomethacin, as shown by increased metabolic activities of cells in the presence of this drug (17).

When induced to convert into the fat-storing phenotype, clusters of GRX cells in the confluent monolayer became polygonal, increased in size, and organized into regular groups with cobblestone appearance. In the perinuclear area they accumulated progressively refringent fat droplets stainable with the Oil Red O (Fig. 2 *A*). The number and the size of fat droplets increased progressively until they occupied most of the cell volume. Fusion of fat droplets into a single central fat vacuole was not observed. Clusters of fat-storing cells were separated by strands of cells with normal elongate morphology. Among these cells, new clusters were progressively formed or individual cells acquired the fat-storing phenotype. In cultures submitted to long-term induction most of cells accumulated lipids (Fig. 3).

Along their conversion into the fat-storing phenotype, GRX cells showed progressive modification of their cytoplasmic structures. In standard conditions, GRX cells displayed a rich fibrillar system subjacent to the cell membrane, with local dense areas characteristic of smooth muscle cells in culture. Their conversion was paralleled with the rounding-up of cells, decreasing their contacts with neighboring cells. A simultaneous reduction of their cytoskeleton and disappearance of distinct stress fibers was observed.

In GRX cells the rough endoplasmic reticulum (RER) was particularly developed, with distended cysternae containing amorphous or fine fibrillar material. Lipocyte conversion induced flattening of cysternae with progressive disorganization of the RER. Ribosomes, originally associated with the RER in the form of circular or spiral polyribosomes, detached and could be found in the cytoplasm as monoribosomes. Glycogen was present in both phenotypes but its content increased with the lipocyte differentiation, and in fully differentiated lipocyte it was occasionally localized in large areas (Fig. 4 *A*).

In the beginning of the lipid droplet formation, an association of the RER with the newly formed lipid inclusions could be observed. Lipid inclusions were surrounded in an early stage of their formation by a continuous membrane, but larger fat droplets were usually devoid of distinct membrane. On tangential cuts of fat droplets, a well-organized layer of parallel and equidistant fibers was observed, forming a regular cage around the droplet. These fibers, compatible in size with intermediate filaments, were in direct contact with the space occupied by lipid (Fig. 4 *B*).

GRX cells contained numerous small dictyosomes, dispersed in the perinuclear area. They were surrounded by small, smooth vesicles. Their size and their number did not increase significantly along the lipocyte conversion. In the submembrane area, plasmalemmal vesicles were abundant, with occasional formation of rosettes around plasmalemmal invaginations, indicating a high pinocytic activity. Larger clathrin-coated vesicles were

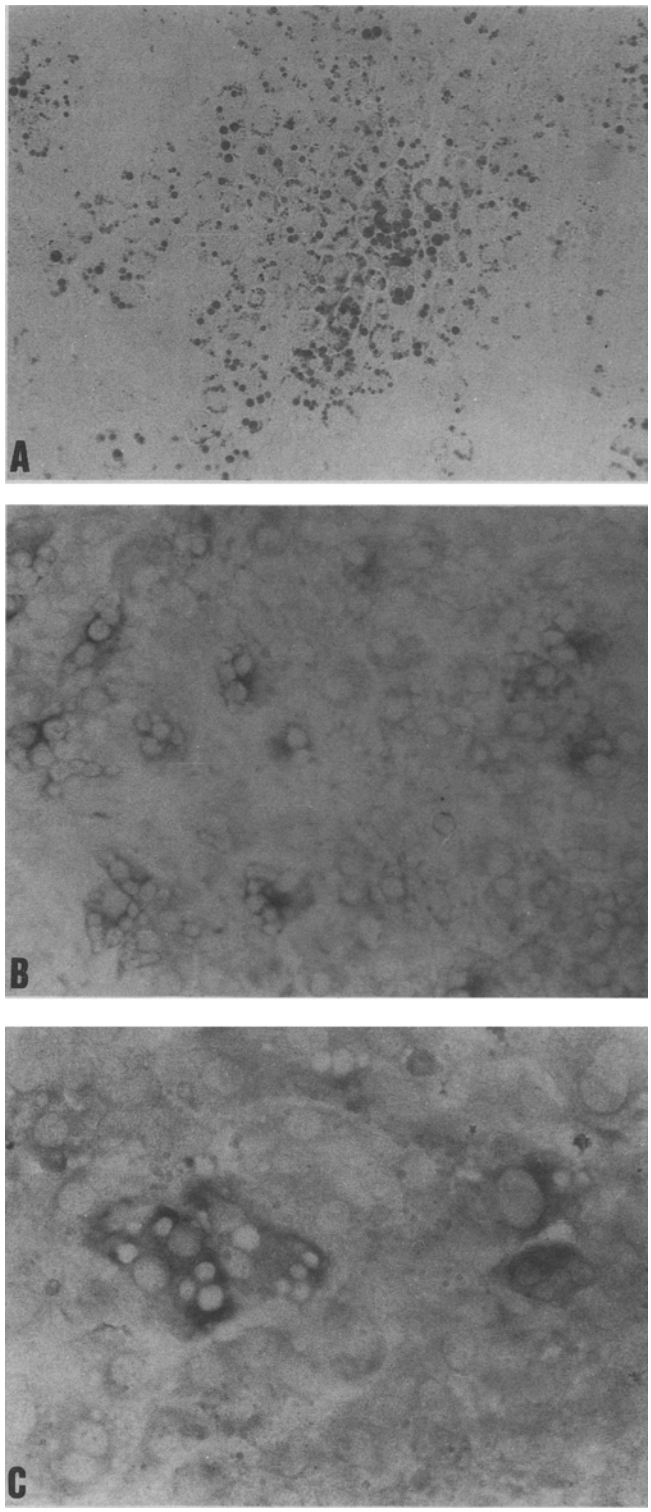

FIG. 2. *A*, Oil red O staining of fat droplets formed in GRX-123 cells after 5 d of induction with insulin and indomethacin. ×200. *B*, glycerol phosphate dehydrogenase. ×500. *C*, nonspecific esterases induction in cells actively engaged in formation of lipid droplets. ×730.

frequent in the peripheral cytoplasm. With the smothering of the cell surface in fully converted cells, plasmalemmal vesicles became less abundant, but clathrin-coated vesicles were present in similar quantities in both phenotypes (Fig. 4 *C*).

In GRX cells mitochondria were elongated or vermiform, with lamellar cristae. Along the lipocyte conversion they became smaller and more compact, with cristae showing tendency toward formation of tubular structures.

Typical desmosomes are characteristic of GRX cells. Their number decreased along the lipocyte conversion.

Extracellular matrix was present among both converted and nonconverted cells. Large collagen fibers were contiguous with basolateral cell membranes. They were associated with fine fibrillar material, ornated with granules suggestive of proteoglycans (Fig. 4 *C*). Discontinuous basement membranelike deposits were observed on both the lower and upper surface of the cell.

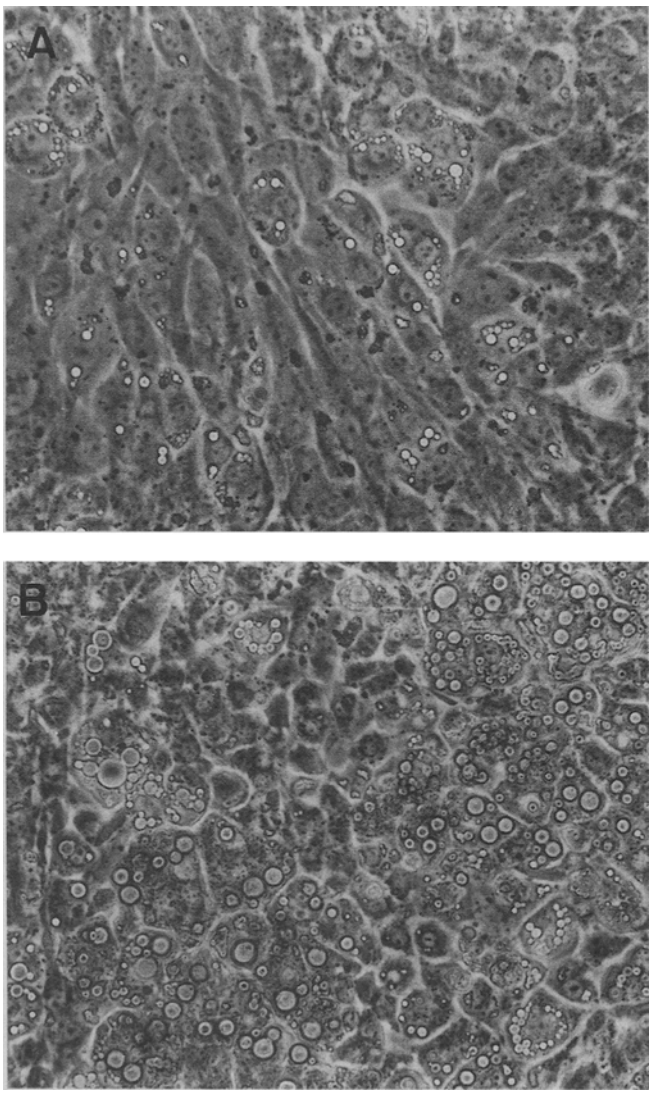

FIG. 3. GRX-123 cells treated with 0.17 μM insulin and 0.12 mM indomethacin, for 5 (*A*) and for 8 (*B*) d. *Note* the formation of spherical lipid droplets. Phase contrast microscopy, ×500.

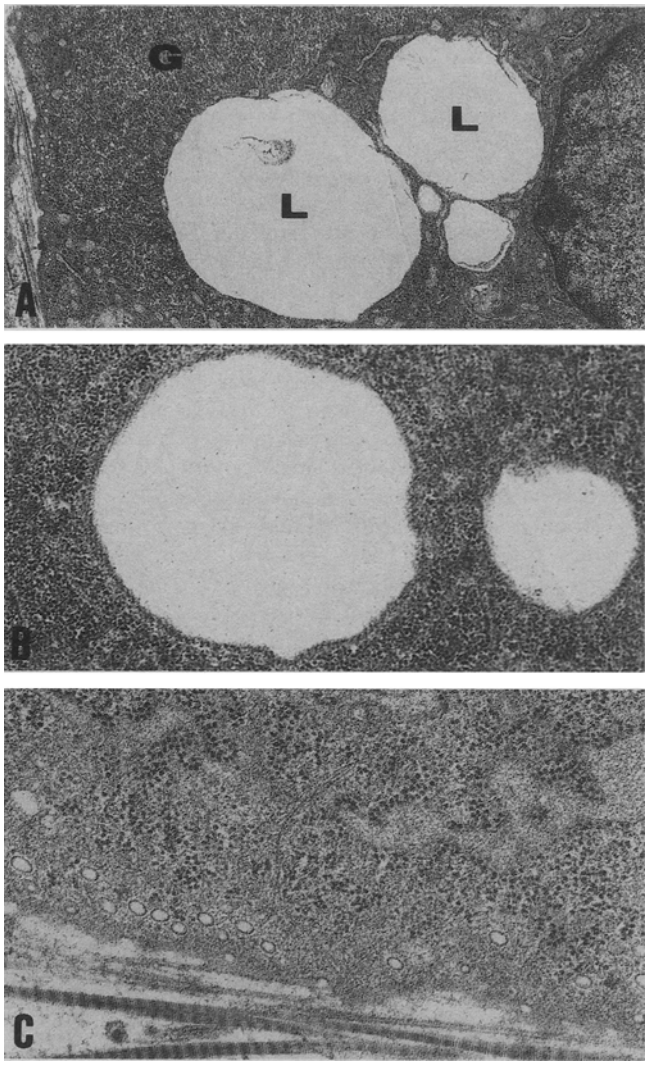

FIG. 4. *A*, lipid droplets (*L*), and glycogen (*G*) accumulation in a GRX cell induced to convert into the lipocyte phenotype, ×2600. *B*, fat droplets in a GRX cell induced to convert into the lipocyte phenotype, ×7800. *Note* the filamentous cage around the droplet. *C*, periphery of a GRX cell with clathrin-coated vesicles and extracellular matrix deposits. ×10 600.

Cytochemical detection of GPDH and esterases showed superposable results. These enzymes could not be demonstrated in GRX cells in the log phase of proliferation. After the induction of the fat-storing phenotype, the enzymes could be demonstrated in clusters of cells containing lipid droplets. With the increase of the size of fat droplets, the intensity of the reaction in their vicinity increased (Fig. 2 *B*, *C*). Lactate dehydrogenase was present in all GRX cells.

The total lipid content of cells maintained under standard conditions expressing the myofibroblastic phenotype and its modification along the induction of the lipocyte phenotype is shown in Fig. 5. A significant difference was observed after only 5 d of induction. A

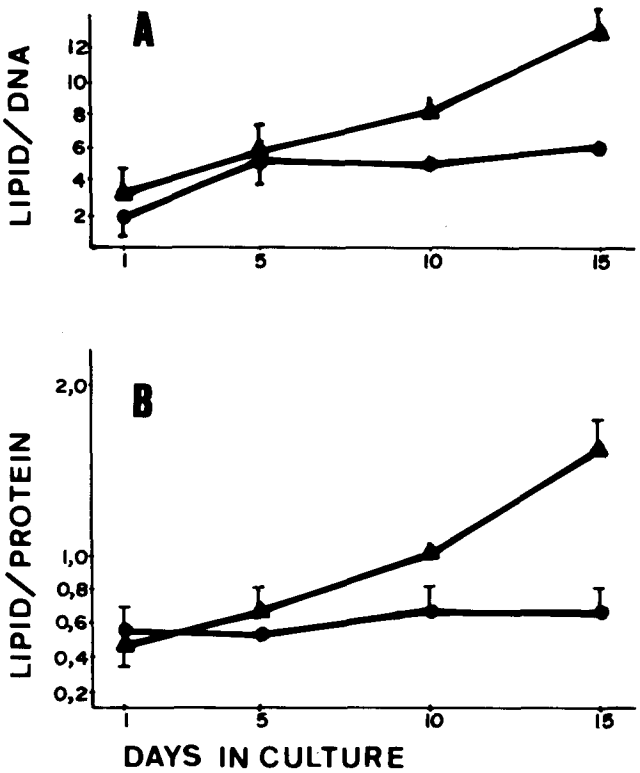

FIG. 5. Lipids accumulated in GRX-123 cells during 15 d of culture under standard conditions (*solid circles*) and in the presence of insulin and indomethacin (*solid triangles*). *A*, lipid content expressed as total lipids/cell DNA content ($\mu g/\mu g$) and *B*, lipid content expressed as total lipids/cell protein content ($\mu g/\mu g$). Mean values and SD (when larger than symbol) for three independent experiments is shown.

slightly higher increase in lipid:protein ratio was observed as compared to lipid:DNA ratio, consistent with the relative decrease of cell structural protein content observed in lipocytes.

The biosynthesis of lipids was monitored by acetate incorporation; it is shown in Fig. 6. Two days after the beginning of treatment with insulin, in the concentrations used, an inhibitory activity could be observed. After the induction of the lipocyte phenotype, from Day 5 on, the activity of indomethacin became predominant and it increased considerably the ratio of acetate incorporation into lipids.

The relative activity of insulin and indomethacin on acetate incorporation is shown in Fig. 7. In the presence of 0.17 μM insulin (Fig. 7 *B*), a low stimulation of acetate incorporation was observed in concentrations of indomethacin lower than 13 nM, but a full effect of indomethacin on lipocyte induction could be observed only in concentrations of 130 μM, and it tripled at 260 μM. In the presence of 130 μM indomethacin (Fig. 7 *A*), a significant stimulatory effect was observed even for low quantities of insulin supplied by the serum (observe in Fig. 7 *A* for 0 on the abscissa, where no insulin was added, and compare with the control values of acetate incorporation in cells with no drugs, Fig. 6). In the presence of indomethacin in this concentration the increase of insulin was paralleled by an increase in acetate incorporation until 1.7 nM. Higher concentrations of insulin supplementing the same quantity of indomethacin had an opposite effect. The cell density observed in the same cultures is also shown in Fig. 7 *A* and *B* by interrupted lines (corresponding to values given on the right scale). Acetate incorporation was inversely proportional to the cell density.

DISCUSSION

Our experimental studies on the GRX cell line give a direct support to the hypothesis of the single connective tissue cell lineage in the liver parenchyma. This lineage can be modulated between the myofibroblastic and lipocyte phenotypes.

The analysis of data shown in Fig. 7 indicates that insulin increases the acetate incorporation into lipids in physiologic concentrations. In concentrations superior to 170 nM, in the range where it acts as insulinlike growth factor, it stimulates proliferation and proportionally decreases the accumulation of fat. We have shown that indomethacin acts as a potent inducer of the lipocyte phenotype, even in very low concentrations of insulin. It

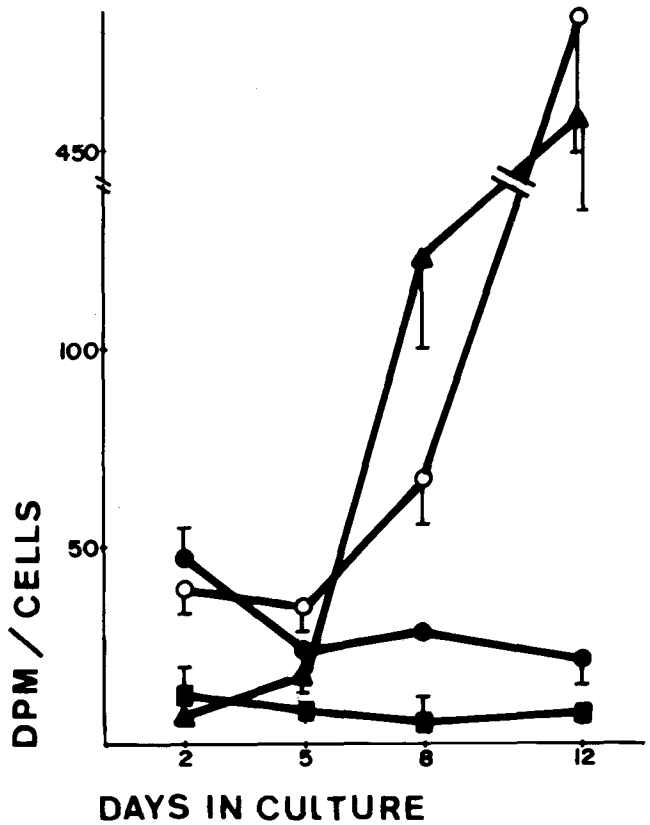

FIG. 6. C^{14}-acetate incorporation into total lipids of GRX-123 cells during 24 h. Cells were previously incubated for 2 to 12 d in standard DMEM containing 5% FBS (*solid circles*) supplemented with 0.17 μM insulin (*solid squares*), 0.13 mM indomethacin (*open circles*) or both (*solid triangles*). Results are expressed as dpm $\times$ $10^{-3}/2 \times 10^6$ cells; mean values and SD (when larger than symbol) for three independent experiments are given.

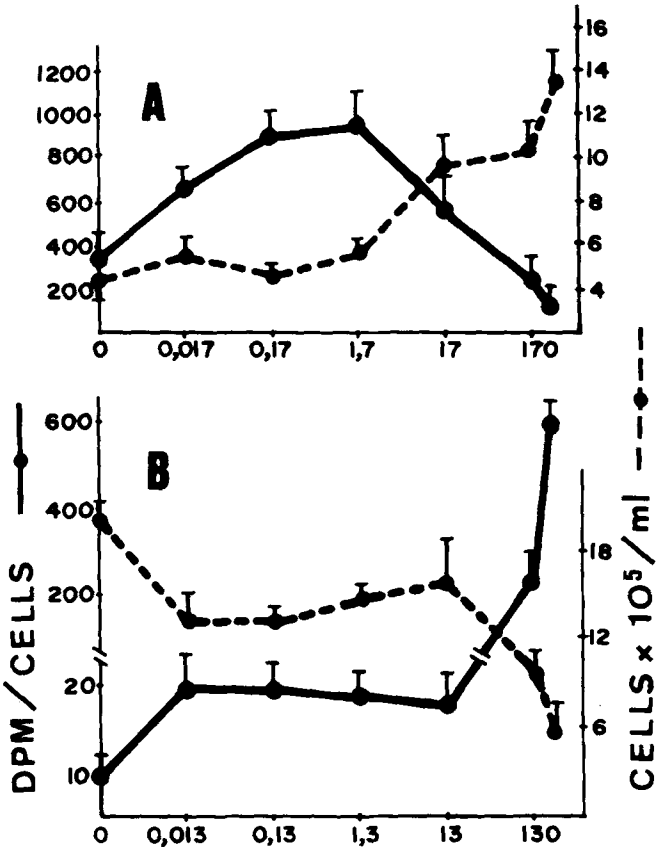

FIG. 7. *A*, effect of increasing insulin (n*M*) and *B*, effect of increasing indomethacin (μM) concentrations on C^{14}-acetate incorporation into total lipids and on proliferation of GRX-123 cells. Cells were maintained for 7 d in standard DMEM supplemented with 5% FBS and the tested drug. They were subsequently incubated for 24 h in the same medium containing radiolabeled acetate. Cells were harvested in 2 ml of BSS, counted, and the total acetate incorporation into cells was quantified by liquid scintillation. Incorporation of acetate is expressed as dpm $\times 10^{-3}/2 \times 10^{6}$ cells (*solid lines*, corresponding to values given on the *left scale*). The number of cells harvested from the same cultures is shown by *broken lines*, corresponding to values given on the *right scale*. Mean values and SD for three independent experiments are given.

slightly increases acetate incorporation in 13 n*M* solution, but the concentrations of indomethacin required for full lipocyte induction are much higher (superior to 13 μM) than those that inhibit prostaglandin production. It is consequently not known if indomethacin activity is due to the inhibition of cyclo-oxygenase pathway and decrease of prostaglandin synthesis, to the accumulation of arachidonic acid that may itself act as a membrane signal transducer (27), or to yet another control at the level of genes expression, as indicated by the fact that the same lipocyte differentiation may be induced by retinol (25) and by sodium butyrate (unpublished results).

GRX cells, proliferating in the log phase, normally do not store triglycerides. After the induction of the lipocyte phenotype, GRX cells accumulate lipids and this corresponds to the de novo synthesis of triglycerides, as demonstrated by incorporation of acetate. Simultaneously, glycerolphosphate dehydrogenase and esterase activity increases from undetectable levels to a very prominent activity around fat droplets in lipocytes. It has been suggested that the esterase activity detected by cytochemistry in cells may be due to the esterolytic activity of glycerolphosphate dehydrogenase (2,32). Consequently, both cytochemical results may indicate activation of essential enzymes in the liponeogenesis. After the induction of lipocyte differentiation with insulin and indomethacin, total lipid accumulation, increase of acetate incorporation into lipids, and cytochemical demonstration of the presence of enzymes involved in lipid synthesis are observed only after a delay of 4 to 5 d. We interpret this delay as a period necessary for synthesis of components of this new metabolic pathway. The conversion of myofibroblasts into lipocytes thus represents the induction of a new phenotype and not simply a metabolic response to a new stimulation.

Cytoplasmic organelle modifications during the induction of lipocyte phenotype are very similar to those observed in preadipocyte differentiation by Novikoff et al. (32). The decrease of cytoskeletal elements is consistent with the rounding up of cells. It corresponds to the decrease of actin synthesis through the control of translatable mRNA, indicating that the shift in the phenotype expression is based in the complex set of modifications in gene expression at the mRNA level (9,37).

The formation of lipid droplets in GRX cell line-derived lipocytes, passing through membrane-bound droplets to those devoid of membranes, and their association with glycogen particles is analogous to that observed in vivo for human and rat Ito cells (38,42). The formation of a specific filamentous cage around lipid droplets has been described so far only in adipogenesis (13,32). Its presence in hepatic lipocytes underlines the close relationship between these two types of connective tissue, fat-storing cells.

Mesenchymal cells that may be converted into fat-storing cells can be grouped in three categories (1):

1. Pre-adipocytes that correspond morphologically and biochemically to fibroblasts and are induced to differentiate into mature adipocytes by insulin.

2. Bone-marrow stroma cells representing precursors of medullar fat-storing tissue, the adipose conversion of which is dependent on steroids.

3. Macrophages that can be induced in vitro to accumulate lipids in less-defined conditions.

The GRX cell line is distinct from these cell groups by their origin, their morphology, their growth behavior, and their ability to secrete extracellular matrix (6). Their conversion into fat-storing cells represents the modulation of hepatic mesenchymal cells between myofibroblast and lipocyte phenotype. They are different from pre-adipocytes that belong to fibroblast cell lineage by their morphologic and biochemical parameters, and should be considered as an independent mesenchymal cell lineage, specific of hepatic parenchyma.

By their perivascular position, their myofibroblastic morphology, their capacity to store retinol, and their activity in fibrogenesis liver connective tissue cells are

similar to pericytes (25). These cells are considered to be a rather undifferentiated mesenchymal cell type that have a wide capacity to differentiate and to participate actively in reparative and regenerative processes. Iyama et al. (20) have proposed that pre-adipocyte have their tissular origin in pericytes. Consequently, both adipocytes and lipocytes may have a similar origin in perivascular cells, that may differentiate in function of the tissue background into adipocytes, hepatic lipocytes, or other mesenchymal cells with similar but distinct functional and morphologic characteristics.

REFERENCES

1. Ailhaud, G. Adipose cell differentiation in culture. Mol. Cell. Biochem. 49:17-31; 1982.
2. Alfonzo, M.; Apitz-Castro, R. A new catalytic activity of *a*-glycerolphosphate dehydrogenase: the enzymatic hydrolysis of *p*-nitrophenyl acetate. FEBS Lett. 19:235-238; 1971.
3. Bhatal, P. S. Presence of modified fibroblasts in cirrhotic livers in man. Pathology 4:139-144; 1972.
4. Bioulac-Sage, P.; Balabaud, C. La cellule perisinusoidale (ou cellule de Ito). Gastroenterol. Clin. Biol. 9:312-322; 1985.
5. Borojevic, R.; Vinhas, S. A.; Monteiro, A. N. A., et al. Liver connective tissue cells isolated from human schistosomal fibrosis or alcoholic cirrhosis represent a modified phenotype of smooth muscle cells. Biol. Cell 53:231-238; 1985.
6. Borojevic, R.; Monteiro, A. N. A.; Vinhas, S. A., et al. Establishment of a continuous cell line from fibrotic schistosomal granulomas in mice livers. In Vitro Cell. Dev. Biol. 21:382-390; 1985.
7. Burton, K. Determination of DNA concentration with diphenylamine. In: Grossman, L.; Moldave, K., eds. Methods in enzymology XII. New York: Academic Press; 1968:163-165.
8. Clement, B.; Emonard, H.; Rissel, M., et al. Cellular origin of collagen and fibronectin in liver. Cell. Mol. Biol. 30:489-496; 1984.
9. Cook, K. S.; Hunt, C. R.; Spiegelman, B. M. Developmentally regulated mRNAs in 3T3-adipocytes: analysis of transcriptional controls. J. Cell Biol. 100:514-520; 1985.
10. Davis, B. H.; Pratt, B. M.; Madri, J. A. Retinol and extracellular collagen matrices modulate hepatic Ito cells collagen phenotype and cellular retinol-binding protein levels. J. Biol. Chem. 261:10280-10286; 1987.
11. DeLeeuw, A. M.; McCarthy S. P.; Geerts, A., et al. Purified rat liver fat-storing cells in culture divide and contain collagen. Hepatology 4:392-403; 1984.
12. Forest, C.; Czerucka, D.; Grimaldi, P., et al. Metabolic characteristics of murine adipocyte precursor clones. In: Angel, A.; Hollenberg, C. H.; Roncari, D. A. K., eds. The adipocyte and obesity: cellular and molecular mechanisms. New York: Raven Press; 1983: 53-64.
13. Franke, W. W.; Hergt, M.; Grund, C. Rearrangement of vimentin cytoskeleton during adipose conversion: formation of an intermediate filament cage around lipid globules. Cell 49:131-141; 1987.
14. Friedman, S. C.; Roll, F. J.; Boyles, J., et al. Hepatic lipocytes: the principal collagen-producing cells of normal rat liver. Proc. Natl. Acad. Sci. USA 82:8681-8685; 1985.
15. Green, H.; Kehinde, O. An established preadipose cell line and its differentiation in culture II. Factors affecting the adipose conversion. Cell 5:19-27; 1975.
16. Grimaud, J. A.; Borojevic, R. Myofibroblasts in hepatic schistosomal fibrosis. Experientia 33:890-892; 1977.
17. Guaragna, R. M.; Moraes, V. L.; Borojevic, R. Liver connective tissue cells: in vitro induction of the fat-storing phenotype. Arq. Biol. Tecnol. 32:36; 1989.
18. Hausman, G. J.; Novakofsky, J.; Martin, R. J., et al. The histochemistry of developing adipocytes in primary stromal-vascular cultures of rat adipose tissue. Histochemistry 80:353-358; 1984.
19. Hirsch, J. G.; Fedorko, M. E. Ultrastructure of human leukocytes after simultaneous fixation with glutaraldehyde and osmium tetroxide and "post-fixation" in uranyl acetate. J. Cell Biol. 38:615-623; 1968.
20. Iyama, K.; Ohzono, K.; Usuku, G. Electron microscope studies on the genesis of white adipocytes: differentiation of immature pericytes into adipocytes in transplanted tissue. Virchows Arch. B Cell Pathol. 31:143-155; 1979.
21. Kawase, T.; Shiratori, Y.; Sugimoto, T. Collagen production by rat liver fat-storing cells in primary culture. Exp. Cell Biol. 54:183-192; 1986.
22. Knook, D. L.; Seffelaar, A. M. Fat-storing cells of the liver. Their isolation and purification. Exp. Cell Res. 139:486-471; 1982.
23. Leo, M. A.; Mak, K. M.; Savolainen, E. R., et al. Isolation and culture of myofibroblasts from rat liver. Proc. Soc. Exp. Biol. Med. 180:382-391; 1985.
24. Mak, K.; Leo, M. A.; Lieber, C. S. Alcoholic liver injury in baboons: transformation of lipocytes to transitional cells. Gastroenterology 87:188-200; 1984.
25. Margis, R.; Borojevic, R. Retinoid-mediated induction of the fat-storing phenotype in a liver connective tissue cell line (GRX). Biochim. Biophys. Acta 1011:1-5; 1989.
26. Margis, M. P.; Domont, G.; Borojevic, R. Collagen biosynthesis during the adipose conversion in the GRX cell line. Arq. Biol. Tecnol. 31:27; 1988.
27. McPhail, L. C.; Clayton, C. C.; Snyderman, R. A potential second messenger role for unsaturated fatty acids: activation of Ca^{2+}-dependent protein kinase. Science 224:622-625; 1984.
28. Minato, Y.; Hasumura, Y.; Takeuchi, J. The role of fat-storing cells in the Disse space fibrogenesis in alcoholic liver disease. Hepatology 4:559-566; 1983.
29. Monteiro, A. N. A.; Borojevic R. In vitro formation of fibrous septa by liver connective tissue cells. In Vitro Cell. Dev. Biol. 23:10-14; 1987.
30. Mourao, P. A. S.; Luz, M.; Borojevic, R. Sulfated glycosaminoglycans synthesis by human smooth muscle cells isolated from different organs. Biochim. Biophys Acta 881:321-329; 1986.
31. Nakano, M.; Worner, T. M.; Lieber, C. S. Perivenular fibrosis in alcoholic liver injury: ultrastructural and histologic progression. Gastroenterology 83:777-785; 1982.
32. Novikoff, A. B.; Novikoff, H. M.; Rosen, O. M., et al. Organelle relationship in cultured 3T3-Ll preadipocytes. J. Cell Biol. 87:180-196; 1980.
33. Rieder, H.; Teutsch, H. F.; Sasse, D. NADP-dependent dehydrogenases in rat liver parenchyma. Histochemistry 56:283-298; 1978.
34. Rudolph, R.; McLure, W. J.; Woodward, M. Contractible fibroblasts in chronic alcoholic cirrhosis. Gastroenterology 76:704-709; 1979.
35. Savolainen, E. R.; Leo, M. A.; Timpl, R., et al. Acetaldehyde and lactate stimulate collagen synthesis of cultured baboon liver myofibroblasts. Gastroenterology 87:777-787; 1984.
36. Sedmak, J. J.; Grossberg, S. E. A rapid sensitive and versatile assay for protein using Coomassie brilliant blue G250. Anal. Biochem. 79:544-552; 1977.
37. Spiegelman, B. V. M.; Green, H. Control of specific protein biosynthesis during the adipose conversion of 3T3 cells. J. Biol. Chem. 255:8811-8818; 1980.
38. Tanuma, Y.; Ito, T.; Shibasaki, S. Further electron microscope studies on the human hepatic sinusoidal wall with special reference to the fat-storing cells. Arch. Histol. Jpn. 45:263-274; 1982.
39. Verrando, P.; Negrel, R.; Grimaldi P., et al. Differentiation of ob17 preadipocytes to adipocytes. Triggering effects of clofenopate and indomethacin. Biochim. Biophys. Acta 663:255-265; 1981.
40. Voss, B.; Rauterberg, J.; Pott, G., et al. Nonparenchymal cells cultivated from explants of fibrotic livers resemble endothelial

and smooth muscle cells from vessel walls. Hepatology 2:19-28; 1982.

41. Williams, I. H.; Polakis, S. E. Differentiation of 3T3-L1 fibroblasts to adipocytes. The effect of indomethacin, prostaglandin E_1 and cyclic AMP on the process of differentiation. Biochem. Biophys. Res. Comm. 77:175-186; 1977.

42. Yamamoto, K.; Ogawa, K. Fine structure and cytochemistry of lysosomes in the Ito cells of the liver. Cell Tissue Res. 233:45-57; 1983.

43. Zerbe, O.; Gressner, A. M. Proliferation of fat-storing cells is stimulated by secretions of Kupffer cells from normal and injured liver. Exp. Mol. Pathol. 49:87-101; 1988.

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