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Growth state-dependent phenotypes of adult hepatocytes in primary monolayer culture

(cell proliferation/retrodifferentiation/α_1-fetoprotein/pyruvate kinase isozymes/glutathione *S*-transferase B)

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ABSTRACT **A proliferation-competent adult rat liver cell monolayer system has been analyzed for tissue-specific functions during its growth cycle. High levels of the adult (L type) form of pyruvate kinase (EC 2.7.1.40) and glutathione *S*-transferase B ("ligandin," EC 2.5.1.18) are observed during the early lag phase; they decline markedly during the logarithmic phase and reappear during the stationary phase. By contrast, elevated levels of the fetal (K type) form of pyruvate kinase and α_1-fetoprotein production appear only after proliferation begins; this pattern diminishes slightly during stationary phase as the adult phenotype is restored. Albumin production continues throughout the entire growth cycle. These *in vitro* findings simulate those observed during hepatoproliferative transitions in the intact animal and, as such, constitute a developmental program for normal epithelial cells in primary culture.**

Many specialized animal cell functions are related to proliferative and developmental states (for review, see ref. 1). Much of this evidence comes from physiological studies of the liver (2–5). For example, the L-isozymic form of pyruvate kinase (EC 2.7.1.40) (6) and glutathione S-transferase B ("ligandin," EC 2.5.1.18) (7) are prominent hepatocyte properties associated with quiescent or adult states whereas the production of α_1-fetoprotein (8) and the K-isozymic form of pyruvate kinase (6) are properties associated with proliferating or fetal states. Temporal aspects of the appearance or disappearance of differentiated properties during transitional states imply that distinct regulatory "programs" exist. However, the molecular and cellular bases of such programs are poorly understood and difficult to study in the intact animal. Hepatocyte tissue cultures offer a more direct approach (9) but versatile adult systems for studying this problem have not yet been obtained (10–14). We now report observations with a novel liver cell culture system (15) which displays differentiation patterns linked to proliferative transitions.

MATERIALS AND METHODS

Reagents. Sera, hormones, collagenase, and tissue culture dishes were obtained as described (15). L-[4,5-^{3}H(N)]Leucine (40–60 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Cycloheximide and reduced glutathione (GSH) were supplied by Sigma (St. Louis, MO).

Primary Liver Cell Cultures. Hepatocytes were isolated from normal Fisher/344 male rats weighing 150–200 g (15). Cells were plated (10^6/35-mm tissue culture dish) into 2 ml of "complete medium" (15), without glucagon or triiodothyronine, and cultured (except as noted below) for 12 days as described (15). Cell proliferation was measured by electronic particle counts of trypsinized attached cells (15, 16). Additional functional and proliferative properties of this system have been reported (9, 15, 17).

Pyruvate Kinase Isozymes. At indicated times, culture media were removed and monolayers (10–20 dishes per point) were washed three times with 2 ml of ice-cold 10 mM phosphate-buffered saline, pH 7.0. Attached cells were scraped with a rubber policeman into 10 mM potassium phosphate, pH 7.0/5 mM magnesium sulfate/0.5 mM dithiothreitol/10% (vol/vol) glycerol and sonicated twice for 30 sec (Branson sonifier set at 50 W). Particulate material was removed by centrifugation at 100,000 × *g* at 4° for 1 hr. Total activity in high-speed supernatants was measured by using the coupled lactate dehydrogenase assay (18). The two different isozyme forms were measured by: (*i*) differential kinetic assay, based upon greater sensitivity of K-isozyme to inhibition by tryptophan (19) and (*ii*) separation on DEAE-cellulose columns that, under the conditions used, bind only the L-isozymic form (20). Supernatant aliquots were applied to columns (10 × 0.6 cm), the K form was eluted with additional buffer, and the bound L form was eluted with buffer containing either 0.5 M KCl or with a 0–0.5 M KCl gradient. Enzyme activity and protein concentrations in collected fractions were determined with 2,4-dinitrophenylhydrazine (21) and the Folin reagent (22), respectively. When kinetic analyses were performed, an additional correction was made because purified K-isozyme is only 90% inhibited by tryptophan at the concentration used (19). This correction was important especially when the fraction of K form exceeded that of the L form. Both column and kinetic methods gave similar results (±10%).

Albumin and α_1-Fetoprotein. At indicated times, culture media were removed and monolayers were washed twice with 2 ml of prewarmed (37°) fresh modified "complete medium" supplemented with 0.4 mM L-arginine but depleted of L-leucine. Duplicate dishes per point then received 2 ml of similar medium containing [^{3}H]leucine (5 μCi/ml) with or without cycloheximide (5 μg/ml) and were returned to the incubator. Controls (i.e., nonspecific "background" subtracted from experimental values) consisted of similar incubations of dishes without cells. Media were collected 24 hr later, centrifuged at 300 × *g* at 21° for 5 min, and stored at −20° until assay. Monolayer lysates were prepared by freezing and thawing (23). Albumin and α_1-fetoprotein concentrations in media or lysates were measured directly by radioimmunoassay or by specific immunoprecipitation of ^{3}H-labeled proteins (24). Both procedures utilize monospecific goat antibodies to purified rat albumin and rat α_1-fetoprotein (23–25). The chemical purity of these antigens and the analyses of radiolabeled immunoprecipitates were checked by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (26) and fluorography (27).

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Abbreviation: GSH, reduced glutathione.

Glutathione *S*-Transferase B. At indicated times, culture media were removed; the monolayers (10–20 dishes per point) were washed three times with 2 ml of phosphate-buffered saline at 4° and then scraped into 2 ml of distilled water. After three cycles of freezing and thawing, 105,000 × *g* supernatants were prepared and analyzed for total protein (22). Ligandin concentrations were measured in supernatant fractions directly by radial immunodiffusion (28) and enzymatically by conjugation of dinitrochlorobenzene with GSH (29).

Data Analysis. For all parameters measured, errors between duplicate cultures averaged ±5–10%; results are averages of 3–10 independent platings. Analysis of variance was performed as described (30).

RESULTS

Proliferation-competent cultures, established in arginine-free medium to suppress nonhepatocyte overgrowth (refs. 15 and 16; Fig. 1), were followed throughout their growth cycle to determine how differentiated functions change during resting and growing states.

Hepatocyte Functions Inversely Related to *In Vitro* Liver Cell Proliferation. Fig. 2 shows pyruvate kinase isozyme levels during the growth cycle expressed as specific activity (per mg of protein) or total activity (per culture). Zero-time cell suspensions (Fig. 2 *bottom*) contained mostly L-isozyme (100% by kinetic assay; 93% by DEAE-column assay), in agreement with morphological findings that initial cell suspensions consist of ~95% hepatocytes (15). L-Isozyme specific activity (0.45 international unit/mg of protein; 10^6 cells ≈ 1 mg of protein) was similar to values reported from other laboratories using different methods for cell isolation and isozyme determination (6, 11, 21).

L-Isozyme levels "per cell" fell abruptly within 6 hr after plating. This was not due to cellular detachment because,

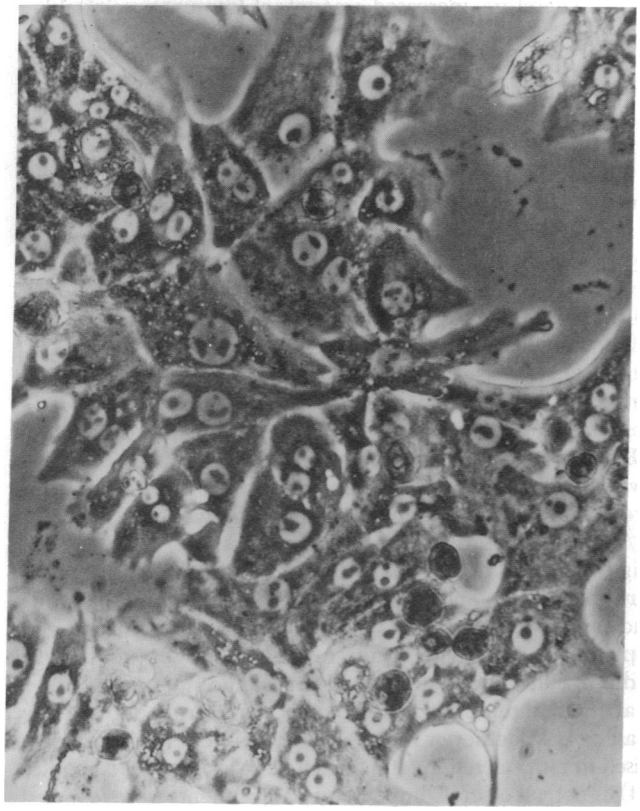

FIG. 1. Phase-contrast photomicrograph of 5-day-old adult liver cell monolayers fixed with neutral buffered formalin. (×172.)

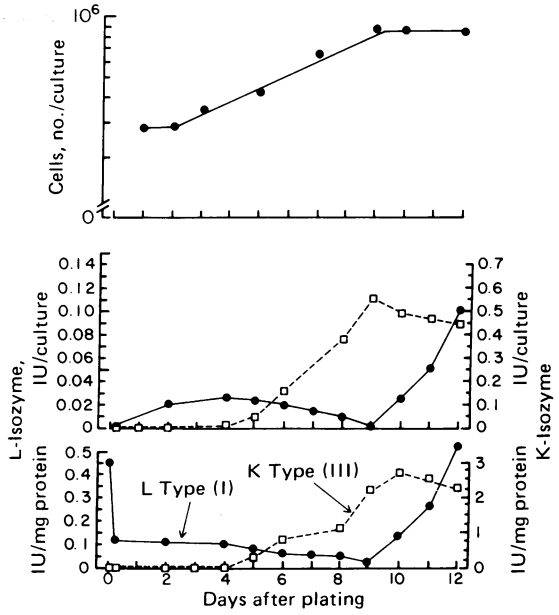

FIG. 2. Pyruvate kinase isozyme levels (*Middle* and *Bottom*) during the growth cycle (*Top*) of adult liver cell cultures. ●—●, L-Isozyme; □ - - - □, K-isozyme. No zero-time point is given in *Middle* because at this time no cells are attached to the culture dish. IU, international units.

during this period (0–12 hr), increasing numbers of viable cells attached to the substratum (not shown) so that the total amount of L-isozyme per culture actually increased. L-Isozyme activity held constant for ~4 days after plating, during the lag and early logarithmic phases, then, a gradual decline began during logarithmic growth, until a minimum was reached 9 days after plating. This secondary decline again represented a decrease in specific activity, not cellular dedifferentiation or detachment, because (*i*) constant or increasing levels of other hepatocyte functions were observed concomitantly (see Figs. 2–5), and (*ii*) L-isozyme activity was not detected in the culture medium. During stationary phase, between 9 and 12 days after plating, L-isozyme specific activity levels returned to initial values and, by day 12, total activity per culture increased in proportion to net cell number increases (~3-fold).

Fig. 3 shows ligandin concentrations or glutathione *S*-transferase B levels per culture during the growth cycle. Day 1 lag-phase monolayers contained ~72 μg of ligandin per mg of protein, similar to fresh liver homogenates (7). This concentration fell 70% as the cell population entered logarithmic phase, between days 1 and 3, despite constant albumin production rates measured by radioimmunoassay of culture fluids (Fig. 4) and without the appearance of detectable ligandin in the culture medium. Ligandin concentrations remained low during the logarithmic phase (through day 7); they were detected catalytically but not by radial immunodiffusion between days 5 and 7. As the stationary phase began, ligandin concentrations increased markedly to ~80% of day 1 levels. In terms of specific activity, however, this increase represented ~33% of values measured on day 1.

Decreases of L-type pyruvate kinase or of glutathione *S*-transferase B activity were not blocked by adding 0.4 mM arginine to the medium. However, if proliferative rates were decreased[e] ~2-fold by culturing cells with 5% (vol/vol) serum,

[e] When adult rat hepatocytes are cultured without added serum they do not proliferate; L-type pyruvate kinase activity does not fall after 1 day under these conditions (31).

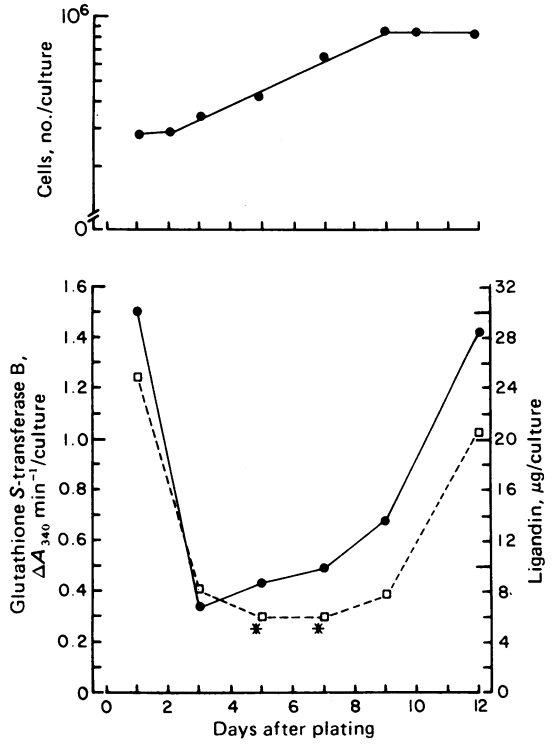

FIG. 3. (*Upper*) Growth curve (*Lower*) Glutathione *S*-transferase B ("ligandin") levels during the growth cycle of adult liver cell cultures. ●–●, Enzymatic activity; □ - - - □, material immunoprecipitable by monospecific goat anti-rat ligandin antiserum. Asterisks, values below immunodiffusion assay detection limits (28).

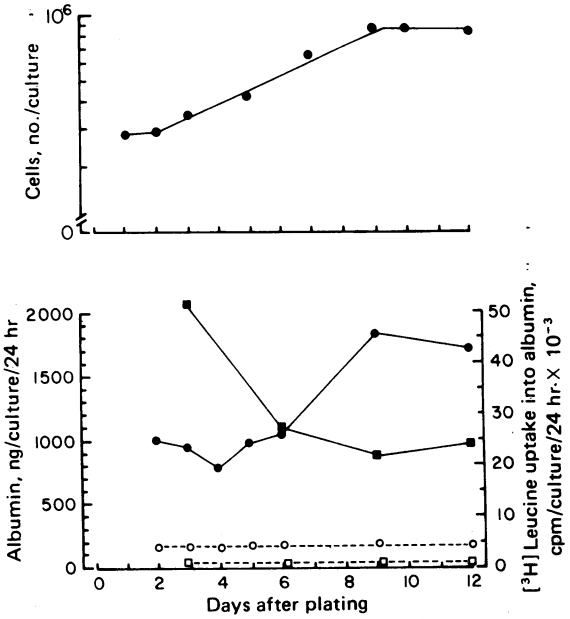

FIG. 4. (*Upper*) Growth curve. (*Lower*) Albumin production during the growth cycle of adult liver cell cultures. ●—●, Radioimmunoassay without cycloheximide; ○ - - - ○, radioimmunoassay with cycloheximide; ■—■, [^{3}H]leucine incorporation into secreted immunoprecipitable protein without cycloheximide; □ - - - □, [^{3}H]-leucine incorporation with cycloheximide.

then a proportional increase in day 5 glutathione *S*-transferase B activity was observed (data not shown).

Hepatocyte Functions Directly Related to *In Vitro* Liver Cell Proliferation. Fig. 2 also shows pyruvate kinase K-isozyme levels during the growth cycle. Zero-time cell suspensions contained traces of K-isozyme (0% by kinetic assay; 5–7% by the DEAE-column assay) possibly due to nonparenchymal cells (15) or to traces of serum which is rich in the K form. Detectable K-isozyme increases were not observed until after the logarithmic phase began (day 5). The onset and elevation of K-isozyme activity was accompanied by the secondary decline to negligible levels of the L form. As the cell population entered stationary phase on day 9, K-form activity reached peak levels disproportionately higher than net increases in cell numbers and considerably higher than peak levels reported for 48-hr regenerating liver (32). K-isozyme levels began to fall slowly by day 10 but, unlike the L form, failed to approach initial values by day 12.

α_1-Fetoprotein production was not detected during the early phases of the *in vitro* growth cycle (Fig. 5). After the logarithmic phase began, α_1-fetoprotein synthesis and secretion increased markedly, as detected by [^{3}H]leucine incorporation into specific immunoprecipitable material, and remained high throughout the stationary phase. Although radioimmunoassayable α_1-fetoprotein was not observed—an expected result given the assay's sensitivity (≥10 ng/ml) and the fact that normal adult regenerating liver produces 0.1% as much α_1-fetoprotein as does fetal liver (33)—similar incorporation results were obtained when cultures were labeled with L-[*guanido*-^{14}C]arginine or [^{3}H]ornithine (data not shown). Secretion of labeled AFP was inhibited ≥95% by cycloheximide (Fig. 5) and was linearly proportional to time and to the quantity of α_1-fetoprotein synthesized and retained intracellularly (data not shown). Therefore, increased amounts of immunoprecipitable labeled α_1-fetoprotein probably reflected net increases in the amounts of newly synthesized α_1-fetoprotein.[f]

Developmental patterns of pyruvate kinase K-type activity or of α_1-fetoprotein production were unaffected either by adding 0.4 mM arginine to the medium or by replacing day 1 culture medium with cell-free conditioned medium from day 3 or day 9 cultures (data not shown). However, precocious α_1-fetoprotein production 1–3 days after plating was observed when adult hepatocytes were plated from animals with proliferating livers—for example, from 70% hepatectomized rats [48–96 hr postoperatively (33)] or from lipotrope-deficient rats (ref. 9; unpublished data). Enhanced α_1-fetoprotein production, without a decreased lag duration, also was observed in proportion to initial serum concentrations ranging from 3–15% (vol/vol) (unpublished data).

Hepatocyte Functions Present throughout the *In Vitro* Liver Cell Growth Cycle. Albumin production rates measured directly by radioimmunoassay ranged between 2.2 and 3.0 μg/24 hr per mg of cell protein throughout the growth cycle (Fig. 4). Under these conditions, secretion was linearly proportional to time and to the quantity of albumin retained intracellularly (data not shown). Cycloheximide blocked the appearance of synthesized albumin in cell lysates (not shown) and into culture media >90%, again excluding the possibility of artifactual release of preformed material from damaged or dead cells. Whether or not prior arginine-deficiency compromised *in vitro* secretion rates, which were less than *in vivo* rates (34), is not yet clear. Compensatory adjustments were revealed by decreased [^{3}H]leucine incorporation into secreted immunoprecipitable albumin despite increased secretion/culture;

[f] When adult rat hepatocytes are cultured on collagen-coated nylon mesh, similar *in vitro* kinetics for AFP production are observed (A. Sirica, Y. Tsukada, and H. Pitot, personal communication).

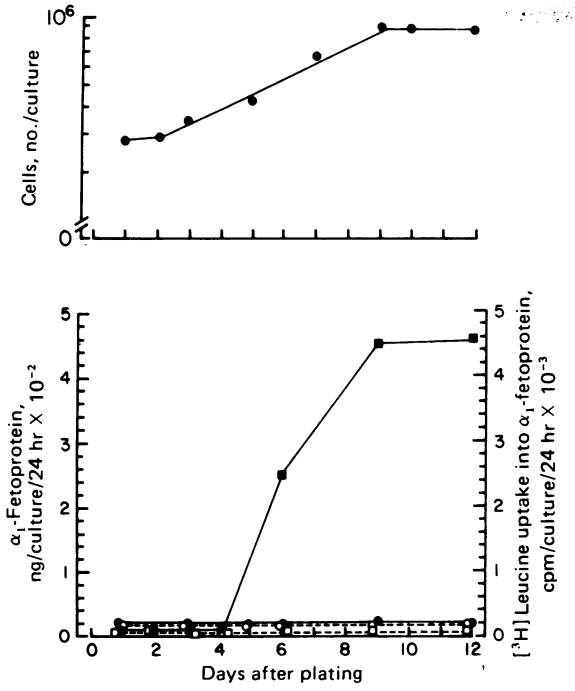

FIG. 5. (*Upper*) Growth curve. (*Lower*) α_1-Fetoprotein production during the growth cycle of adult liver cell cultures. ●—●, Radioimmunoassay without cycloheximide; ○ - - - ○, radioimmunoassay with cycloheximide; ■—■, [^{3}H]leucine incorporation into secreted immunoprecipitable protein without cycloheximide; □ - - - □, [^{3}H]leucine incorporation with cycloheximide.

by contrast, [^{3}H]ornithine (15) or L-[*guanido*-^{14}C]arginine incorporation into immunoprecipitable albumin showed coordinate relationships to albumin production measured by radioimmunoassay.

DISCUSSION

We have measured a set of prominent hepatocyte properties during the growth cycle of a novel proliferation-competent adult rat liver cell culture system (15). The levels of adult functions such as the L-isozymic form of pyruvate kinase and the anion-binding cytosol protein glutathione *S*-transferase B ("ligandin") (7) decrease more than 70–80% during resting-to-growing transitions but increase abruptly as proliferative rates decline. Conversely, the levels of fetal functions such as the K-isozymic form of pyruvate kinase and α_1-fetoprotein production increase during logarithmic growth and, like the regenerating liver, are maintained for some time after proliferative rates decline (6, 33). This pattern simulates hepatic functional alterations in animals undergoing hepatoproliferative transitions; it is unaltered by arginine-supplemented or conditioned medium.

Previous observations (15) indicate that day 5 logarithmic phase cultures contain at least 75% hepatocytes, of which 40% also synthesize DNA. By day 12, the cell population doubles (refs. 15 and 17; Fig. 2) but the hepatocyte fraction remains ≥80% as judged either by [^{3}H]ornithine radioautography (9, 15) or by specific immunofluorescent staining for albumin (9, 23) or ligandin (unpublished observations). This suggests that a large fraction of functioning hepatocytes traverse the entire cell cycle at least once in this system. Although the present findings do not permit conclusions concerning relationships between phenotypic changes and cell-cycle progression of individual cells, preliminary observations suggest that one or more serum factors involved with controlling growth rates also may be involved with regulating developmental changes. That fetal functions fail to reach resting levels after 4 days of stationary phase may be due to normally slow decay mechanisms (32), to partial cell "cycle" arrest of an hepatocyte subpopulation, or (with respect to the K-isozyme) to the presence of a secondary nonhepatocyte population[g] that survives arginine-deficient growth conditions by contact crossfeeding (16). Further studies are necessary to distinguish among these possibilities.

The concomitant disappearance and reappearance of hepatocyte functions during hepatoproliferative transitions has been termed "retrodifferentiation" (35). The reversibility of these phenomena (see Figs. 2 and 3), together with high rates of albumin production throughout the *in vitro* growth cycle, virtually excludes common tissue culture artifacts such as plating "trauma," dedifferentiation, or cell death which complicated the interpretation of earlier attempts to study this problem in nonproliferating liver cell systems (11–14).

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[g] The presence of such a population has not been measured directly but is inferred from the fact that, during culture, 5–20% of the attached cells fail to stain detectably with hepatocyte-specific immunofluorescent markers (9, 23) and are only marginally labeled as visualized by [^{3}H]ornithine radioautography (9, 15).

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