Proc. Nat. Acad. Sci. USA
Vol. 68, No. 10, pp. 2396–2400, October 1971

Production of Albumin and Other Serum Proteins by Clonal Cultures of Normal Human Liver

(human embryonic cells/human hepatocytes/human liver cells/cytodifferentiation)

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Communicated by James D. Ebert, July 14, 1971

ABSTRACT **Clonal cell strains have been isolated from normal human liver. These cells, while resembling fibroblasts morphologically, function as hepatocytes, as shown by their ability to synthesize and secrete an antigen identical to human serum albumin. Human diploid and aneuploid cell lines from nonliver sources do not exhibit this property. The spectrum of serum proteins synthesized varied from clone to clone and cell line to cell line.**

Many attempts have been made to isolate functionally-intact parenchymal cells from normal liver that could be serially propagated in culture (1–5). The results of these efforts have been disappointing in that during the early phase of culture, islets of epithelial cells presumed to be parenchymal were replaced by nonspecialized fibroblastic cells of mesenchymal origin. Two explanations have been given for these observations (6): Either normal differentiated parenchymal cells from the liver were altered as a result of the conditions of culture (5, 7) or they were lost by selective overgrowth of ubiquitous nonspecialized cells (3, 6–8). In discussing these alternatives, most investigators have tended to equate functional with morphological characteristics. However, morphology may be a poor index of function in cell culture.

Retention of tissue-specific structures and biochemical functions by normal cells in culture is now well documented in several systems (9–11). Similarly, clonal strains of rat hepatoma cells have been shown to retain liver-specific properties (12–14). In contrast, cell strains from normal liver have not been reported to retain liver-specific properties. More recently, epithelial cell clones have been isolated from newborn and adult rat liver* (15). Fetal cells from mouse parenchyma also have been grown as mass cultures by the use of a selective, chemically defined medium to discourage the growth of other cell types. This line, now predominantly triploid after three years in culture, still has liver function†. These findings prompted renewed efforts to isolate cell strains from normal human liver capable of liver-specific function.

Experiments to be described will show that clonal strains from normal fetal and adult human liver exhibit liver-specific functions such as synthesis of albumin, although in appearance they resemble fibroblasts rather than epithelial cells as expected for liver parenchyma. Evidence for the synthesis of other serum proteins by liver and other control cell lines is also presented.

Abbreviations: RID, radioimmunodiffusion; HE/SM, human embryonic skin and muscle; HEK, human embryonic kidney; HLX, human fetal liver; ID, immunodiffusion; RID, radioimmunodiffusion.

* Coon, H. G., *J. Cell Biol.*, **39**, 29A (1968).

† Waymouth, C., H. W. Chen, and B. G. Wood, *In Vitro*, **6**, 371A (1971).

MATERIALS AND METHODS

Cells

Clones were isolated from primary cultures of normal human liver taken either by surgical biopsy or at autopsy, and from a monolayer culture (6th passage) of human fetal liver (HLX), which was a gift from Dr. K. F. Soike. Other cell lines used included WI38, Chang liver, KB (from American Type Culture Collection), HG286 (from Dr. J. German), HE/SM (human embryonic skin and muscle from Microbiological Associates, Inc.), HEK (human embryonic kidney, isolated in this laboratory), and HeLa. Cells were preserved in liquid nitrogen.

Cloning and maintenance of cell cultures

Methods were similar to those used by Coon to clone rat liver (15). Cell suspensions were derived from minced liver by progressive digestion with CTC (0.1% collagenase, 0.1% trypsin, 1% chicken serum in Ca^{++}- and Mg^{++}-free Hanks' balanced salt solution). Petri dishes (100 mm, Falcon Plastics, Los Angeles, Calif.) received 10^4–10^6 trypan blue-excluding cells in 6 ml of growth medium, which consisted of Ham's F12 medium (16) with double the recommended concentrations of amino acids and sodium pyruvate, penicillin G, K^+ (100 units/ml), Kanamycin (100 μg/ml), Amphotericin B (6.25 μg/ml), and 1.5 g/l extra $NaHCO_3$ supplemented with 17% fetal-calf serum (Flow Labs.). The efficacy of each lot of serum used was tested by its ability to support clonal growth of a rat liver epithelial cell (Clone 9) isolated in this laboratory. Cultures were incubated in a humidified atmosphere at 36.5°C continuously flushed with sufficient CO_2 to maintain the medium at pH 7.4. Cultures were fed thrice weekly. Single, well-isolated colonies were encircled with (Dow-Corning) silicone-coated porcelain cylinders (17), rinsed with Ca^{++}-, Mg^{++}-free Hanks' solution, suspended with CTC, and transferred to a 10-cm Petri dish containing 6 ml of medium. When the cells had become confluent, they were either used for an experiment or frozen and stored in liquid nitrogen.

Analysis of samples

For identification of serum proteins, cultures were incubated with F12 medium without serum (see *Figure Legends*). The culture fluid was filtered (0.45-μm membrane filter), dialyzed versus GC buffer (0.3% glycine, 0.05% sodium citrate,

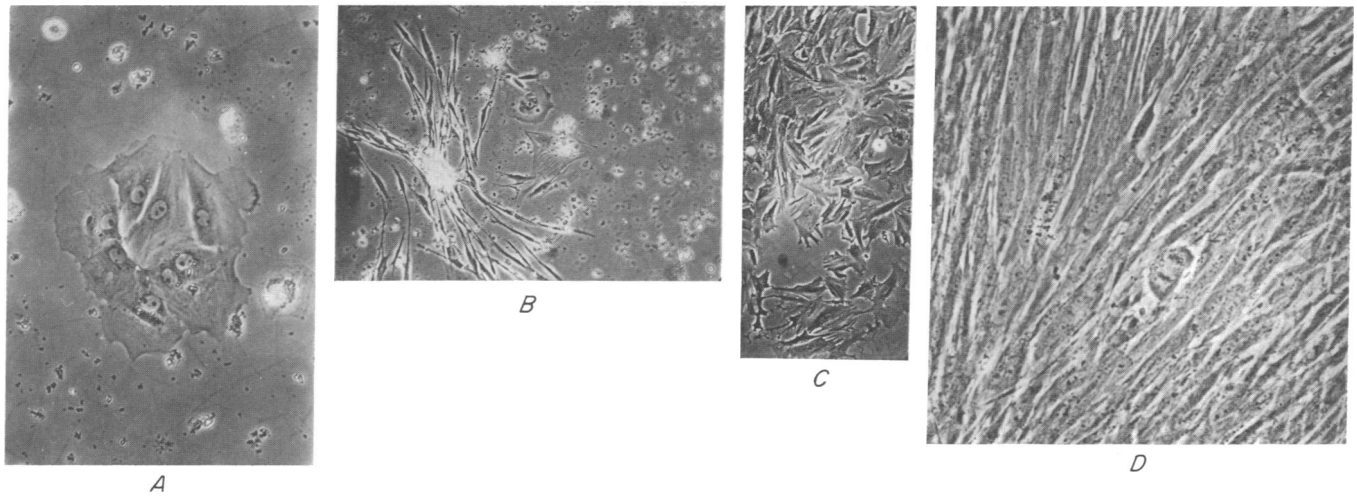

Fig. 1. Human liver cells in culture; (*A*) epithelial colony of cells after six days in culture. Two nearby cells are preparing for division (×175). Note binucleate cell on lower right; (*B*) fibroblastic colony after 6 days in culture (×87.5). Note two different cell types nearby; (*C*) Edge of a colony that apparently arose from epithelial cells (×87.5), (*D*) Mass culture of fetal liver cells, HLX, at tenth passage (×87.5).

0.02% sodium chloride, and 0.2% glucose), lyophilized, dissolved in distilled water (0.2–0.5 ml), and analyzed by double diffusion in agarose (18), immunoelectrophoresis (19), disc electrophoresis (20), or radial immunodiffusion (21). Antisera to human serum proteins produced in goats, horses, or rabbits were obtained from Hyland Labs., Los Angeles, California, or Behring Diagnostics, Woodbury, New York. Precipitin patterns were photographed by incident light after overnight development at room temperature. Protein was measured after precipitation with 10% trichloroacetic acid (22).

RESULTS

Isolation of clonal strains from human liver

Epithelial, fibroblastic, and other cell types were observed in all primary cultures (Fig. 1). After a week or so, the epithelial colonies tended to spread out and assume a more fibroblastic form. Fig. 1*C* depicts the edge of such a colony shortly before it was subcultured. In early experiments in which several lots of each type of serum were compared, it was found that fetal-calf serum was superior to horse, calf, or human serum and that relatively high concentrations were needed (10% or more). In contrast, for rat liver cells, 5% fetal-calf serum appeared to be optimal (15). Primary cultures differed widely in plating efficiency depending on the condition of the specimen. On the average, one in 10^4 cells yielded a viable colony. Four clones (C1–C4) were isolated from a mass culture of fetal liver (HLX) (Fig. 1*D*). All were fibroblastic like the parental strain. Under favorable conditions, the plating efficiency of HLX was 50–70%. Its average doubling time was 24 hr during logarithmic growth. Primary cultures were established from 8 of 13 liver specimens ranging in age from a 2-day-old premature infant to an adult stabbing victim of unknown age. From 5 of these, 37 colonies were isolated and studies on 12 of these colonies are reported here. Thus far, no attempt has been made to determine the longevity of these strains since cells at early passage have been available from frozen stocks. However, one strain (HLX) has been passed at least 30 times (about 70 generations). A preliminary study of its chromosomes by Dr. Raju Chaganti (personal communication)‡ indicates that it has the normal human male karyotype. Examination of 14 metaphase plates revealed that 12 were 2n and 2 were 4n. This could have been expected, since binucleate cells are common in the liver.

Assay for human plasma protein synthesis by radioimmunodiffusion (RID)

Cultures of 10 fibroblastic human liver clones, from an epithelial aneuploid human liver line (Chang), and from human cells of nonliver origin (epithelial and fibroblastic) were examined for plasma antigens (Table 1). Liver strains incorporated [^{14}C]leucine into insoluble antigen–antibody complexes containing fibrinogen, transferrin, α_2-haptoglobin, albumin, prealbumin, and α_1-antitrypsin. Individual clones, even from the same liver, differed with respect to the number and species of proteins produced. The other five antigens listed in Table 1 were synthesized by various cell types, as reported by others (23, 24), and were not unique to liver. In addition, none of the cell lines listed synthesized hemopexin, ceruloplasmin, α_1-lipoprotein, or plasminogen (not shown). Chang cells produced five antigens by RID, which included fibrinogen. Its synthetic pattern is clearly distinct from those of HeLa and the other nonliver lines (see *Discussion*).

Experiments in which radioimmunoprecipitation is used to study protein biosynthesis must be interpreted cautiously (25), since uptake of isotope can occur by binding of the amino acid itself or by coprecipitation with a newly-made nonspecific protein (26). The former possibility was ruled out by a control experiment in which [^{14}C]leucine was incubated with human plasma. No isotope was incorporated into the antigen–antibody complex. To circumvent the possibility of nonspecific labeling inherent in the use of the radioimmunological method, direct immunoprecipitation was employed. Since this required greater quantities of antigen, cells were grown on a larger scale.

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TABLE 1. *Radioimmunodiffusion of media from human liver clones and human cell lines (from other organs)**

	Liver clones†										Cell lines						
	Fibroblastic										Epithelial				Fibroblastic		
	Fetal				Infant (7 Wk.)				Adult		Liver	Kidney	Carcinoma		Skin		Lung
Antigen	C1	C2	C3	C4	3	4	6	7	20	21	CG‡	HEK	Hela	KB	S§	S/M¶	W138
Fibrinogen	+	−	+	+	+	+	+	+	−	−	+	−	−	−	−	−	−
Transferrin	+	−	+	+	+	−	+	+	−	−	+	−	−	−	−	−	−
Albumin	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
Prealbumin	‖NT				+	+	+	+				−	−	−	−	−	−
α_1-Antitrypsin					−	−	+	−									
α_2-Haptoglobin	−	−	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−
β_2-Glycoprotein-1	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	+
α_1-Acid Glycoprotein	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+
α_2-Macroglobulin	−	+	+	+	+	+	+	+	−	−	+	−	−	−	−	+	+
β_1-Lipoprotein	+	−	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+
C'3-Complement	−	−	+	+	+	+	+	+	−	−	+	−	−	+	+	+	+

* Confluent Petri-dish cultures were washed three times with F12 medium (minus leucine) and 4 ml of F12 containing [^{14}C]leucine (5 μCi/ml, 300 Ci/mol) were added. After 24 hr of incubation, the medium was removed, dialyzed versus GC buffer containing 1 mM L-leucine, lyophylized, and dissolved in 0.1–0.2 ml of water. Sample wells of Ouchterlony plates were filled with 5 μl of appropriately diluted carrier human serum followed by three 5-μl applications of the sample. Monospecific antisera versus human plasma proteins were added to adjacent wells. When precipitin patterns had developed, the plates were washed for three days in PBS, 1 day in distilled water, dried, and exposed to Kodak Royal Pan film for 2 weeks. Autoradiographs coincident with carrier patterns were scored as +, definitely labeled or −, unlabeled (PBS, phosphate-buffered saline).

† Sub-clones 3, 4, 6, and 7 of HFL2 were isolated from a 7-week-old infant with congenital brain deformity at autopsy; sub-clones 20 and 21 of HL8 from adult stabbing victim; C1–C4 from HLX. ‡ Chang liver cells. § Skin fibroblasts. ¶ Skin and muscle fibroblasts. ‖ Blank spaces indicate not tested because of unavailability of antisera at the time.

Detection of serum proteins by direct immunodiffusion (ID)

Medium was harvested from confluent cultures grown in roller bottles. Concentrates of these samples were compared with human serum by double diffusion. Six of the nine normal liver strains and the Chang line produced and secreted an antigen identical to human albumin (Table 2, Fig. 2), whereas the non-liver lines did not. This antigen has the electrophoretic mobility of albumin, as shown by immunoelectrophoresis (Fig. 3) and by acrylamide disc electrophoresis (data not shown). Only one of three clones from HLX (Table 2) produced albumin; HLX itself synthesized only α_2-macroglobulin. HL7, a mass liver culture, produced albumin, C'3, and C'4. All the nonliver cell lines tested produced α_2-macroglobulin, KB and W138 cells produced C'3, and C'4 was only produced by KB cells.

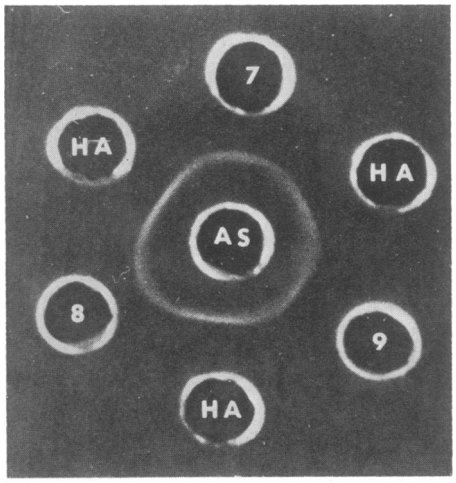

Fig. 2. Identity of an antigen from liver cell culture with human serum albumin by Ouchterlony method. Inner well contains goat anti-human albumin. Outer wells: HA, human albumin (0.6 mg/ml); No. 7, Chang cells; No. 8, HLX, No. 9, HU-13.

The fact that HLXC4 and Chang cells made albumin observable by ID (Table 2), but not by RID (Table 1), suggested that the bottle method of culture favored albumin synthesis. To test this possibility, one bottle and 17 plates were seeded with HLXC4 cells so that cell number, surface area, and medium volumes were equivalent in the two systems. The cells produced about ten times as much albumin in the one bottle (11 mg/g of cell protein) as in the 17 plates (1–2 mg/g of cell protein). Both systems contained similar amounts of cell protein (1.5 mg) at the end of the experiment.

DISCUSSION

Application of cloning techniques has made it possible to isolate cell strains from normal human liver that retain specific function, as demonstrated by their production of albumin and other serum proteins characteristic of liver. These cells are stable in monolayer culture for more than 50 generations and clone with high plating efficiency. They can be recovered to yield reproducible populations for experimental use. Even with cloning, all strains isolated so far have been fibroblastic. There appeared to be a transition from epithelial to fibroblastic morphology during the early stages of clona-growth. Previous workers interpreted this transition as resulting from overgrowth of "fibroblasts" or as loss of specialized function. Possibly both mechanisms may play a roll; i.e., morphological transition and overgrowth could operate concurrently in crowded cultures.

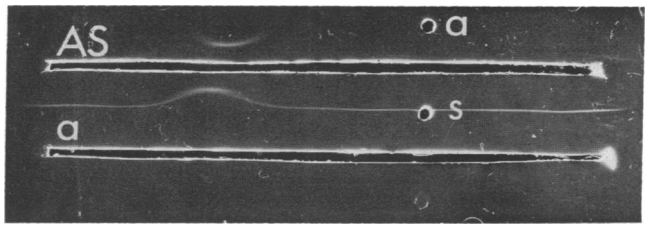

Fig. 3. Immunoelectrophoresis of liver cell culture fluid by Osserman modification (19). Upper well: (*a*), human albumin, 0.2 mg/ml; lower well; (*s*), culture fluid from HLXC4. After electrophoresis, troughs were cut and filled with goat anti-human albumin (*AS*) and human albumin, 0.2 mg/ml (*a*). Note reaction of identity between sample, *s*, and precipitin line between *a* and *AS*.

Control experiments show the methods used to demonstrate serum protein synthesis to be reliable. In RID, carrier human plasma did not bind [^{14}C]leucine (25). Since radioactive samples from different clones and cell lines were developed with the same carrier, differential results between individual liver clones and cell lines also make artifacts of nonspecific binding unlikely. Direct immunoprecipitation without isotopic labeling avoided these problems. However, the possibility of crossreactions between antisera and residual antigens from the fetal calf serum used to grow the cells had to be considered, even though serum had been omitted from the test media. The antisera used in these experiments gave a single band with human serum and none with fetal calf serum.

A number of serum proteins were detected by RID but not by ID. This could reflect a greater sensitivity of RID. On the other hand, more cultures synthesized albumin when tested by ID than by RID. Since samples were collected from plates for RID and from bottles for ID, it appeared that albumin synthesis might be favored by the bottle method. When this possibility was tested, 10 times as much albumin, as assayed by ID, was produced in bottles as in plates. The reason for this is unknown. However, more efficient gas exchange or exposure to nutrients in bottles could be involved. A study of the serum proteins produced by cells originating from tissues other than liver supported the conclusion that only liver cells made albumin. However, certain liver clones did not produce albumin, and the overall pattern of protein synthesis varied from clone to clone.

The observation that Chang cells synthesize human albumin was unexpected, especially since the authenticity of this cell line has been questioned. Gartler (27) has contended that Chang and other cell lines are contaminated by HeLa cells on the basis of glucose-6-phosphate dehydrogenase isozyme patterns. Our results show that HeLa does not produce human albumin (28), whereas Chang does. The two cell lines also differ morphologically. These observations strongly indicate that at least this line of Chang cells (obtained from American Type Culture Collection) originated from human liver. Thus, organ-specific markers can be useful in characterizing cell lines. It may also be of some interest that synthesis of α_2-macroglobulin by aneuploid and diploid human cell lines was demonstrated by both ID and RID methods. This represents true synthesis rather than nonspecific coprecipitation as has been thought (29).

Experiments in which the biosynthetic capacity of the entire liver is examined cannot be interpreted on the single cell level because of the presence of cell types other than the parenchymal cell, including the sinusoid-reticuloendothelial system, connective tissue elements, and bile duct cells. Since clonal cultures retain many of the biosynthetic capacities of their progenitors, this method can be used as an amplification technique to study the properties of individual cells. It can be asked, for example, whether all cells of a given type possess the same biosynthetic potential. Data presented above support, but do not prove, the hypothesis that individual clones may exhibit such diversity. The role of environmental factors will have to be examined in greater detail before this is clear.

The availability of strains of differentiated human liver cells makes possible their use *in vitro* in somatic-cell hybridization studies, virus studies, experiments on chemical and viral hepatocarcinogenesis, and investigation of other liver functions. It may even be possible to isolate strains capable of producing rare plasma components *in vitro*, such as antihemophilic factor.

TABLE 2. *Identity of culture-media components with human-serum antigens**

Cell strains	Albumin† Day 1	Albumin† Pooled	α_2-Macroglobulin† Day 1	α_2-Macroglobulin† Pooled	β_{1A}/β_{1C}-globulin† (C'3) Pooled	β_{1E}-globulin† (C'4) Pooled
Liver						
HLX	—	—	—	I	—	—
HLX-C1	—	—	—	—	—	—
HLX-C2	—	—	—	—	—	—
HLX-C4	I	I	—	—	—	—
HU-13	N.D.	I	N.D.	—	N.D.	—
HU-16	N.D.	I	N.D.	—	N.D.	—
HFL-1	—	I	—	—	—	—
HFL-2	I	I	—	—	—	—
HL7	—	I	—	—	I	I
Chang	—	I	—	—	I	I
Nonliver						
KB	—	—	—	I	I	I
HeLa	—	—	I	I	—	—
WI38	—	—	I	I	I	—

* Cell suspensions were prepared by treatment of nearly confluent cultures with CTC. Roller bottles (Bellco Glass Co. #7000) containing 100 ml of medium F12 supplemented with 17% fetal calf serum, were equilibrated with 5% CO_2 in air. Each bottle, after receiving 3×10^6 cells, was rotated at 1.5 rpm in an incubator at 36.5°C. When confluent, bottles were washed three times and fed with 150 ml of medium F12 without serum. Media were collected daily and those collected on days 2–4 for each sample were pooled. The reaction of concentrated samples with monospecific antisera was compared to that with human serum by the Ouchterlony method and scored as follows: —, negative; I, reaction of identity with human antigen; N.D., not done; † The goat antisera used did not crossreact with fetal calf serum.

We thank Dr. H. G. Coon for helpful discussions, Dr. K. F. Soike for fetal liver cells, and Miss Heddy Zirin and Mrs. Harriet Snitkin for expert technical assistance. This work was supported by grants from the National Institutes of Health (GM 17146 and HE 09011), and was presented in preliminary form to the Tissue Culture Association (*In Vitro*, 6, 371A [1971]).

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