PRIMARY CULTURES OF HEPATOCYTES ON HUMAN FIBROBLASTS

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SUMMARY

Parenchymal hepatocytes isolated from adult rats were cultured on three types of collagen-containing substrata: collagen-coated plates, collagen membranes and confluent diploid human fibroblasts. Hepatocytes on the latter two substrata maintained characteristic morphology for at least 10 days in culture, whereas degenerative changes (cell death and formation of multinucleated hepatocytes) and growth of nonparenchymal elements were seen after 5 days in cultures on collagen-coated plates. Parallel findings were seen on basal and induced levels of cytochrome P-450 and NADPH-cytochrome C reductase. The basal levels of cytochrome P-450 were not measurable after day 3 in hepatocytes cultured on collagen-coated plates, whereas measurable levels were maintained in the hepatocytes cultured on the other two substrata. Addition of phenobarbital or methylcholanthrene at day 5 in culture caused an increase in cytochromes P-450 and P-448, respectively, only in hepatocytes cultured on collagen membranes and confluent fibroblasts. Analogous results were seen for the enzyme NADPH-cytochrome C reductase. The similarities in performance between hepatocytes on collagen membranes and on human fibroblasts show that a continuous collagen-containing substratum is important for optimal performance of hepatocytes in primary culture. The possible importance of cultures of hepatocytes on human fibroblasts for carcinogenesis studies is discussed.

Key words: hepatocytes; cytochrome P-450; NADPH-cytochrome C reductase; collagen-coated plates; floating collagen membranes; confluent fibroblasts.

INTRODUCTION

Several systems of primary culture of hepatocytes have been introduced in the last decade and have been applied to studies related to hepatic physiology and chemical carcinogenesis (1-7). In primary cultures of hepatocytes on rigid substrates (plastic or collagen coated) the cells remain viable for 4-8 days and most hepatospecific markers decrease sharply with increasing age of the cultures (1,2,4,7). A notable case has been that of cytochrome P-450, which in the above systems, when nutrient media supplemented only with insulin or serum are used, decreases to very low levels within 24 hr and is not measurable after 72 hr (8). When hepatocytes are maintained on collagen membranes (3) they remain viable for more than 20 days (9). They also maintain decreased but measurable (10) and inducible (11) levels of reactive (12) cytochrome P-450. Similar improvement in cell viability on collagen membranes has been seen for mammary epithelial cells (13). The reasons for the differences in behavior of hepatocytes in the two different types of substrates (rigid plastic or collagen-coated plate versus pliant-collagen membrane) are not clear. It has been postulated that because of the proximity of the collagen membrane to the surface of the medium the exchange of O$_2$ and CO$_2$ with the atmosphere is facilitated (3). The importance of adequate oxygenation for cells in culture, including hepatocytes, has been demonstrated by McLimans et al. (14). In order to investigate further the role of the substratum in determining the behavior of hepatocytes in culture we compare in this report the maintenance of microsomal functions in hepatocytes cultured on collagen-coated...
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plates, collagen membranes and on confluent, diploid human fibroblasts. Confluent fibroblast cultures are analogous to collagen membranes in the sense that they allow the hepatocytes to attach on a continuous substratum that contains collagen. They are also analogous to collagen-coated plates in the sense that they are a rigid substratum and that the attached hepatocytes are at the bottom of the culture plate.

In a recent report (15,16) it was shown that when chemical procarcinogens were added to combined cultures of hepatocytes and nonconfluent populations of an established Chinese hamster cell line (V-79), mutations could be scored in the V-79 cells. Similar results were also seen in mixed hepatocyte-rat fibroblast cultures (17). These studies emphasize that cocultivation systems of hepatocytes with cell lines may become of value in development of screening systems for detection of mutagens. In this regard analysis of the properties of combined cultures of hepatocytes and human fibroblasts assumes particular importance.

MATERIALS AND METHODS

All nutrient media and fetal bovine serum were purchased from Grand Island Biological Company (Grand Island, New York). Chemicals, hormones and organic buffers were purchased from Sigma Chemical Company (St. Louis, Missouri). Male Sprague-Dawley rats (200-300 g in weight) were purchased from Charles River Company (Wilmington, Massachusetts) and were used as a source of hepatocytes.

Hepatocytes were prepared by perfusion of the rat liver according to the method previously described (1,3). After the perfusion the hepatocytes were suspended in Leibovitz-15 medium and washed by centrifugation (50 x g) three times. The cell pellet of the third centrifugation was suspended in Waymouth medium (MB 752/1) supplemented with bovine insulin (10^{-7} M), fetal bovine serum (5%), penicillin (100 μg per ml), bovine serum albumin (Fraction V) (2 g per l), HEPES (N-2-hydroxyethyl-piperazine-N’2-ethane-sulfonic acid) (15 mM), MOPS (morpholinopropane sulfonic acid) (10 mM) and TES (N-tris [hydroxymethyl] methyl-2-aminoethane sulfonic acid) (10 mM).

The use of the organic buffers HEPES, MOPS and TES was necessary to prevent the rapid drop in pH seen in the medium of the combined cultures of hepatocytes on human fibroblasts when medium not supplemented with organic buffers was used. This rapid decrease in pH occurs probably because the metabolic products of both hepatocytes and the human fibroblasts are accumulating in the same culture vessel. Media were adjusted to pH 7.4 after the addition of the organic buffers and prior to the addition of sodium bicarbonate and, without further pH adjustment, were sterilized by filtration under pressure.

The hepatocytes were suspended at a cell density of 6 x 10^6 cells per ml. Four milliliters of media with hepatocytes were inoculated in 60-mm Falcon tissue culture plates pretreated in each case to achieve the proper substrate as described below. Four hours after inoculation of hepatocytes the medium was changed, and 4 ml of fresh medium was added. The same medium (Waymouth MB 752/1 supplemented as above, including the organic buffers) and the same volume (4 ml), was used for all cultures regardless of the substratum, in order to allow comparison. The cultures were maintained in an atmosphere of 5% CO₂ and 90% humidity at 37°C, and the medium was changed daily. Whenever necessary, phenobarbital or methylcholanthrene were added daily to the media; phenobarbital was dissolved directly into the medium. Methylcholanthrene was added from stock solutions of 2 x 10^{-3} M in dimethyl-sulfoxide (DMSO). Final concentration of DMSO in the medium was 1:200. This concentration was found not to have any significant effects on the parameters tested in this report for cultured hepatocytes (11,18). Collagen membranes and collagen-coated plates were prepared as previously described (3).

Diploid human fibroblasts (Cell line Detroit 550, passage 12) were obtained from the American Type Culture Collection (Rockville, Maryland). They were cultured in Ham’s medium F-12 supplemented with penicillin (100 μg), streptomycin (100 μg) and 15% fetal bovine serum. For the experiments described in this report, cells at a passage number of 14–18 were used. The cultures of fibroblasts were used as substratum for hepatocytes when fibroblasts were completely confluent and all the surface of the 60-mm plate was covered (1.6–1.8 million fibroblasts per plate). The media of the fibroblast cultures were aspirated immediately prior to the inoculation of hepatocytes. The fibroblasts were not in any way treated with chemical fixatives or irradiated prior to the addition of hepatocytes.