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CONTINUED HIGH ALBUMIN PRODUCTION BY MULTICELLULAR SPHEROIDS OF ADULT RAT HEPATOCYTES FORMED IN THE PRESENCE OF LIVER-DERIVED PROTEOGLYCANS

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SUMMARY Adult rat hepatocytes formed floating multicellular spheroids, when they were cultured with proteoglycan fraction isolated from rat liver reticulin fibers. Cells in the spheroid showed only low growth activity. Albumin production by the spheroids increased up to 1.5 μg/μg DNA/day (180 μg/mg Protein/day) during the first 6 days and remained constant thereafter. In contrast, the albumin production by the monolayer markedly decreased after 4 days. The spheroid culture appears to be more suitable than the monolayer in studying differentiated functions of adult hepatocytes.

Recent developments in cell culture technology have made possible long survival times and better maintenance of differentiated functions of hepatocytes in primary cultures (1). In particular, hepatotropic growth factors in combination with other soluble factors allow for the maintenance of normal adult hepatocytes in serum-free medium (2). Furthermore, the extracellular matrix and its components are used as culture substrates to improve the attachment and survival of the cultured hepatocytes (3,4). Better maintenance of the differentiated function are known to be achieved by monolayer cells in high cell density culture also, in which cell growth is more suppressed (5). However in spite of the various efforts, full maintenance of the differentiated functions of hepatocytes *in vitro* is still difficult. For example, in most cases reported albumin secretion gradually decreased when the cells were cultured (6).

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; EGF, epidermal growth factor.

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Hepatocytes in vivo are surrounded by framework of connective tissue, namely reticulin fibers, to which the hepatocytes are probably anchored. We have recently isolated and partially characterized the matrix components of the reticulin fibers of rat liver (7). We found also that a proteoglycan fraction isolated from the reticulin fibers was effective for the formation of multicellular spheroids of adult rat hepatocytes in the primary culture (7).

In this paper, we describe the different fractions of the matrix exerts different effects on the cell and that the spheroid cells in high cell density are poorly proliferative and highly productive for albumin.

Materials and Methods

Extraction of Collagens, Glycoproteins and Proteoglycans --- The reticulin fibers were isolated from livers of Sprague-Dawley rats as described previously (7). Prior to the extraction, the reticulin fibers were pulverized in liquid nitrogen. Collagens were extracted from the ice powder of fiber fragments by pepsin digestion in acetic acid, and isolated by salt precipitation as described by Rojkind, et al. (8). The collagens finally solubilized in 0.02 M acetic acid were used as the collagen fraction in which about 10 mg of protein was recovered from the livers of 50 rats. Glycoproteins and proteoglycans were extracted with 4 M guanidine-HCl from the ice powder of fiber fragments and separated by a column chromatography on DEAE cellulose as described (7). A fraction unbound to the column, was positive for only neutral sugar, while a fraction that bound and eluted with around 0.2 M NaCl was positive for the both. After collagens in the unbound fraction was removed by precipitation in 0.02 M phosphate buffer, the supernatant was used as the glycoprotein fraction, in which about 10 mg of protein was recovered from 50 rats. The hexuronic acid positive fraction, in which about 2 mg of protein was recovered from 50 rats, was used as the proteoglycan fraction.

Chemical Analysis --- Non-collagenous protein was determined by the dye binding method (9) with bovine serum albumin as a standard, and collagenous protein after modification (10) with type I collagen (Nitta Gelatin) as a standard. Neutral sugar was determined by the anthron reaction (11) with galactose as a standard, and hexuronic acid by the carbazole reaction (11) with glucuronic acid as a standard.

Culture Substrate --- The dishes used were 35-mm polystyrene plastic dishes (Falcon, 3001). After dialysis against PBS for 48 h, a 0.2 ml aliquot each of the three fractions whose chemical composition was shown in Table 1, was applied to a dish. The dishes were air-dried, sterilized under UV light for 16 h and then washed with sterile PBS just before initiation of the cultures.

Cultures --- Hepatocytes were isolated from Sprague-Dawley rats, weighing about 150-200 g, by the collagenase-liver-perfusion method (12). About 99 % of the cells obtained were hepatocytes, as determined by phase-contrast microscopy. The medium used was a hormone-defined medium (2), with a minor modification. The medium comprised William #E medium supplemented with 10 μg/ml insulin, 0.1 μM copper ($CuSO_4.5H_2O$), 3 nM selenium (H_2SeO_3), 50 pM zinc ($ZnSO_4.7H_2O$), 50 ng/ml EGF (Takara Shuzo),

Table 1. Chemical composition of extracellular matrix component fractions used for dish coating

Fraction	Protein (μg)	Neutral Sugar (ng)	Hexuronic Acid (ng)	Identified Molecules
Collagens	100	ND	ND	Type I and III collagens
Glycoproteins	60	12	<1	Fibronectin Laminin
Proteoglycans	60	2	40	Dermatan sulfate Heparan sulfate

ND; not done
Value are the amount in 0.2 ml aliquot that was applied to a dish.

50 μg/ml linoleic acid, 100 U/ml penicillin, 100 U/ml streptomycin and 1 μg/ml fungizone. Culture was initiated by inoculating 5 x 10^5 cells per a dish containing 1.5 ml medium, and maintained under a humidified atmosphere of 5% CO_2 and 95% air, at 37°C. The medium was changed after 4 h, and then at 2 day intervals for 2 weeks.

Thymidine Uptake and Autoradiography --- Cells were labeled with 0.3 μCi/ml [^{3}H]-thymidine (New England Nuclear Corp., 20 Ci/m mol), for 24 h. Assays for the radioactivity incorporated by cells and autoradiography with the labeled cells was carried as described (13). The labeling index was calculated with the formula, [number of cells with nuclei showing silver staining] / [5 x 10^3 cells scanned] x 100.

Determination of Albumin --- Albumin in the medium was measured by Laurell rocket immuno-electrophoresis (14) using 1% agarose gel containing 50 μl 2-fold diluted anti-rat albumin rabbit serum (Cappel) and rat albumin (Cappel) as standards.

Measurement of DNA --- The amount of DNA in the cultured cells was fluorometrically determined by the method of Hinegardner (15) using calf thymus DNA as a standard.

Results and Discussion

Hepatocyte cultures were initiated by seeding the single cell suspension into dishes coated with the collagen fraction, the glycoprotein fraction, and the proteoglycan fraction. Hepatocytes attached and spread in the three dishes during the initial few days. Remarkable morphological difference became apparent after 4-5 days of culture; the hepatocytes assembled to form monolayers in the collagens-coated dishes, multilayer islands in the glycoproteins-coated dishes, and floating multicellular spheroids in the proteoglycans-coated dishes (Fig. 1). The viability of cells in these cultures were critically examined by the Trypan Blue dye exclusion and previously by histological approaches (7,16). We observed that cells assembled to monolayers, multilayers were viable for 2 weeks and become rapidly dead thereafter. The spheroid cells were proved still viable even

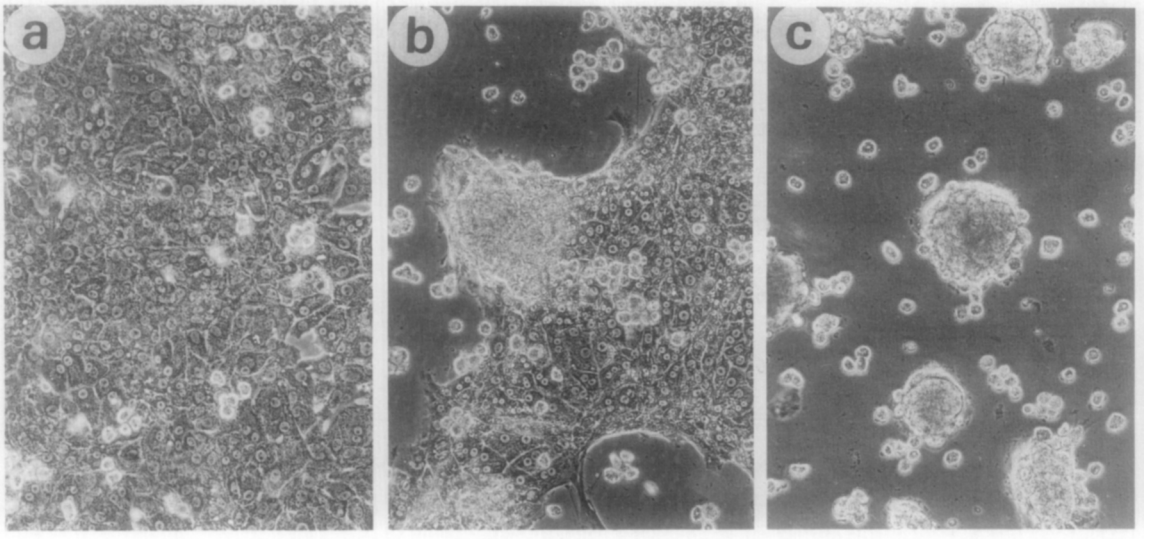

Fig. 1. Monolayer, multilayer islands and spheroids induced by the collagen, glycoprotein and proteoglycan fractions of rat reticulin fibers. Photographs were taken at 4 days of culture in dishes coated with the collagen fraction, a; the glycoprotein fraction, b; and the proteoglycan fraction, c.

after 2 weeks of culture, but the number of spheroids appeared to decrease gradually without marked change of their diameter (Fig. 2). This is partially due to the fact that the floating spheroids were easily aspirated when the medium was changed.

In pararel with the decrease of cell number, the amount of DNA in spheroid culture decreased gradually. No cell proliferation was observed in spheroid cultures as examined thymidine uptake (Table 2). On the other hand, the DNA amount in monolayers and multilayers increased 1.2 - 1.4 fold during the initial 1 week of culture and decreased thereafter (Table 2). Proliferative ability of the two cultures were also shown by a ^{3}H-thymi-

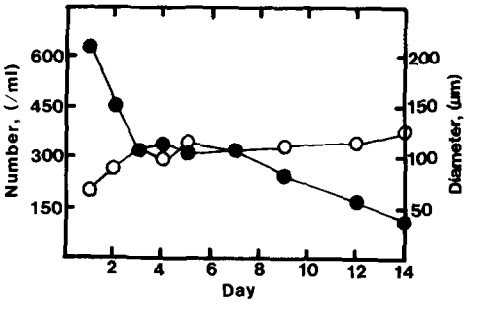

Fig. 2. Change in diameter and number of spheroids during culturing. The number (●) and the diameter (○) of spheroids was determined under phase-contrast microscopy. The values are averages of 3 experiments.

Table 2. Albumin production by hepatocytes in monolayers, multilayers and spheroids

Day	Monolayer			Spheroid		
	DNA (μg/dish)	Albumin (μg/ml)	Alb/DNA/Day (μg/μg/day)	DNA (μg/dish)	Albumin (μg/ml)	Alb/DNA/Day (μg/μg/day)
2	16.6	12.5	0.56	10.0	12.0	0.90
4	23.4	9.0	0.29	12.5	15.1	0.91
6	21.0	5.8	0.21	9.1	17.2	1.42
8	22.6	6.6	0.22	6.0	12.2	1.52
10	29.3	4.9	0.13	5.4	11.5	1.60
12	25.3	1.4	0.04	4.8	11.0	1.72
14	11.7	0.8	0.05	4.0	9.0	1.69

Cultures were initiated by seeding 5 x 10^5 cells per a 35-mm dish. The values were average of 3 experiments.

dine uptake assay (Table 3). Thus, cells in multicellular spheroids in which the cell density was much higher than in the monolayers, are less proliferative probably due to contact inhibition of growth. It is interesting to note that the growth of fetal liver cells (17) and various transformed cells (18) are not suppressed even under high cell density such as in spheroids.

Since the growth ability and the expression of differentiated functions are known to be in a reciprocal relationship (5), albumin secretion, a typical differentiated phenotype of adult hepatocytes, were investigated comparatively in the three cultures. The amount of albumin secreted in the medium was not markedly different for the initial few days, and became different after 4 days. In monolayers, the amount of albumin con-

Table 3. Proliferative ability of hepatocytes in monolayer, multilayer and spheroid cultures

Day	Monolayer		Spheroid	
	Uptake (cpm)	Index (%)	Uptake (cpm)	Index (%)
1	363	-	184	-
2	2.788	34	1.150	1
3	2.921	35	411	1
4	801	-	90	-
5	-	4	-	0
6	527	-	15	-
7	638	-	141	-
8	-	3	-	1
9	441	-	20	-

"Uptake" indicates the radioactivity incorporated in cells / a dish and "Index" does the value of nuclear labeling index. The values were an average of 3 experiments. -; not done.

tinuously decreased, while in spheroids it was maintained in a relatively high level (Table 2). Multi-cell layer formed in the presence of glycoproteins gave intermediate values (data not shown). The difference as to albumin production became more apparent when the amount of production by a unit cell was compared. As shown in Table 2, albumin production by the monolayer cells rapidly decreased to almost none for 2 weeks, while the value by the spheroid cells increased up to about 1.5 μg/μg DNA/Day, (180 μg/mg protein/day), around at day 4 when the formation of the spheroid became completed, and was maintained at the level thereafter. This level of albumin production is similar to that observed in the liver in vivo (19). As above, monolayers and the spheroids are the two typical assemblings of adult rat hepatocytes in primary culture; they are in reciprocal relationship as to proliferating ability and albumin production. The biological features of spheroids resemble to those of hepatocytes in vivo, and spheroid cultures is expected to be valuable in molecular analysis of various differentiated functions of hepatocytes.

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References

1) Reid, L.M., Narita, M., Fujita, M., Murray, Z., Liverpool, C. and Rosenberg, L. (1986) In Research in isolated and cultured hepatocytes (A. Guillouzo, and C. Guguen-Guillouzo. Eds.), pp. 225-258, Jhon Libbey Eurotext Ltd/INSERM, London.
2) Enat, R., Jefferson, D.M., Ruiz-Opazo, N., Gatmaintan, Z., Leinwand, L.A. and Reid, L.M. (1984) Proc. Natl. Acad. Sci. 81, 1411-1415.
3) Rojkind, M., Gatmaintan, Z., Mackensen, S., Ponce, P. and Reid, L.M. (1980) J. Cell Biol. 87, 255-263.
4) Sawada, N., Tomomura, A., Sattler, C.A., Sattler, G.L. and Kleinman, H.K. (1987) In Vitro Cell Dev. Biol. 23, 267-273.
5) Nakamura, T., Yoshimoto, K., Nkayama, Y., Tomita, Y. and Ichihara, A. (1983) Proc. Natl. Acad. Sci. 80, 7229-7233.
6) Crane, L.J. and Miller, D.L. (1977) J. Cell Biol. 72, 11-25.
7) Shinji, T., Koide. N. and Tsuji, T. (1988) Cell Struct. Funct. 13, 179-188.
8) Rojkind, M., Giambrone, M.A., and Biempica, L. (1979) Gastroenterology 76, 710-719.
9) Bradford, M. (1976) Biochemistry 72, 248-258.
10) Duhamel, R.C., Meezed, E. and Brendel, K. (1981) J. Biochem. Biophys. Methods 5, 67-74.
11) Spiro, R.G. (1966) In Methods Enzymol. Vol. VIII. pp. 3-26, Academic Press, New York.
12) Seglen, P.O. (1976) In Methods in Cell Biology, (D.M. Prescott, Ed.), Vol. XIII, pp. 29-83. Academic Press, New York.
13) Nakamura, T., Tomita, Y. and Ichihara, A. (1983) J. Biochem 94, 1029-1035.

14) Laurell, C.B. (1966) Anal. Biochem. 15, 45-52.
15) Hinegardner, R.T. (1971) Anal. Biochem. 39, 197-201.
16) Asano, K., Koide N. and Tsuji, T. (1989) J. Clin. Electron Microscopy 22, (in press).
17) Landry, J., Bernier, D., Ouellet, C., Goyette, R. and Marceau, N. (1985) J. Cell Biol. 101, 914-923.
18) Sutherland, R.M. and Durand, R.E. (1984) Recent Results Cancer Res. 95, 24-49.
19) Peter, T., Jr, and Peter, J. C. (1972) J. Biol. Chem. 254, 10095-10101.