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LONG TERM PRODUCTION OF ACUTE-PHASE PROTEINS BY ADULT RAT HEPATOCYTES CO-CULTURED WITH ANOTHER LIVER CELL TYPE IN SERUM-FREE MEDIUM

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SUMMARY : Three acute-phase proteins, haptoglobin, α_2-macroglobulin and hemopexin, as well as albumin, have been measured daily in the hydrocortisone-supplemented serum-free medium of pure and mixed cultures of adult rat hepatocytes for 5 and 20 days respectively. Whereas plasma protein production rapidly declined in pure culture, it remained relatively stable when hepatocytes were co-cultured with rat liver epithelial cells. In the latter cultures, an early stimulation of albumin and α_2-macroglobulin secretion was observed. In addition, four other plasma proteins, fibrinogen, α_1-acute-phase protein, α_1-acid glycoprotein and α_1-antitrypsin were shown by immunodiffusion to still be produced by day 20 of co-culture. These results suggest that hepatocyte co-cultures represent a suitable model for studying the mechanism which controls synthesis of plasma proteins, including acute-phase proteins by liver cells.

Most of the plasma proteins except immunoglobulins are synthesized by the liver. Among them is the group of acute-phase proteins whose plasma concentrations increase during inflammation by a mechanism which is still poorly understood (1).

In vitro experimental models have been used to study the inflammation process and biosynthesis of acute-phase proteins. These models, which include liver slices (2) or isolated and cultured hepatocytes (3-8), suffer from inherent limitations mainly due to their short lifespan and/or rapid phenotypic alterations. Indeed, it has been well documented that even in primary culture, hepatocytes do not retain their differentiated state : they exhibit a shift towards a more-foetal state within a few days (9).

Recently we devised a method which permitted adult rat hepatocytes to maintain, *in vitro*, their capability of producing a high level of albumin for several weeks (10). This method is based on the association of parenchymal cells with an epithelial cell line derived from rat liver. This

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led us to look at whether, in co-culture, adult rat hepatocytes retained their ability to secrete other plasma proteins and particularly acute-phase proteins. We report here that three rat acute-phase proteins, haptoglobin, α_2-macroglobulin and hemopexin (11) were produced, like albumin, at a relatively constant level for several weeks in such a co-culture system maintained in serum-free medium. Moreover, we found that various other acute-phase proteins were also still detectable by day 20 of co-culture.

MATERIAL AND METHODS

Cell cultures : Pure and mixed cultures of adult rat hepatocytes were prepared and maintained essentially as described (10). After isolation by collagenase perfusion of the liver (12), parenchymal cells were seeded in Ham F-12 medium containing insulin and 10 % foetal calf serum. Three hours later, the medium was discarded and replaced by serum-free medium with or without untransformed liver epithelial cells derived from newborn Fisher rats (13). Then , the serum-deprived medium supplemented with hydrocortisone hemisuccinate was renewed every day. The concentrations of the corticosteroid were 7×10^{-5} and 3.5×10^{-6} M in pure and mixed cultures respectively (10).

Antigens : Rat haptoglobin (14), α_2-macroglobulin (15) and hemopexin (11) were purified according to methods previously described. Rat albumin was obtained from rat serum by a three step procedure. First, an ammonium sulphate precipitation (60 - 75 % saturation) was used. Then, the precipitate dissolved in, and exhaustively dialyzed against 0.1 M acetate buffer, pH 6.0, containing 1 M Nacl, 1 mM $MgCl_2$, $CaCl_2$ and $MnCl_2$, was submitted to affinity chromatography on a concanavalin A (Con. A) Sepharose column equilibrated with the same buffer. Finally, the fraction not retained by Con A, dialyzed against 0.05 M phosphate buffer, pH 7.0, was submitted to anion exchange chromatography on a DEAE A 50 column (20 cm ; diameter 3.5 cm) equilibrated in the same buffer. Proteins retained on the gel were eluted by a linear concentration of phosphate buffer pH 7.0, from 0.05 to 0.2 M followed by an elution with 0.3 M phosphate buffer, which gave pure albumin as revealed by immunoelectrophoresis and 7 % polyacrylamide gel electrophoresis.

Antibodies : Specific antisera directed against haptoglobin, α_2-macroglobulin, hemopexin and albumin were raised in rabbits as previously described (16).

Assays : Protein concentrations in culture media were determined by an electroimmunoassay (17) using 1 % agarose (HSB Litex, Copenhagen, Denmark). The supporting medium was prepared by melting 1 % agarose in Tris (hydroxymethyl) amino-methan 36.6 mM-barbital 12.2 mM buffer, pH 8.6, containing 1.5 mM sodium azide and 0.15 mM calcium lactate. Glass plates (20.5 x 11 x 1.5 cm) were covered with 34 ml of agarose containing antiserum. The amounts of specific antiserum used were 0.44, 0.22, 0.66 and 0.44 $\mu l/cm^2$ for the determination of haptoglobin, α_2-macroglobulin, hemopexin and albumin respectively.

The amounts of medium layered for protein determination were also different. For albumin, 5 µl of culture medium were layered in a 2.5 mm diameter well punched out in the gel ; for hemopexin, 10 µl of medium were layered in two successive wells ; for haptoglobin and α_2-macroglobulin, 40 µl of medium were layered in a 2.5 x 15 mm channel. All the samples were layered under an electric field of 11 volts/cm. The temperature of the gels was kept constant at 9-10°C during electrophoresis performed in a Berhing electrophoresis apparatus connected to variable power supply delivering constant voltage for 16-18 hr. After electrophoresis, the assay plates were blotted with Whatman n° 1 filter paper, rinsed in 0.6 % Nacl (w/v) for 30 min

and directly dried on a heating plate at 50°C. Immunoprecipitates were stained with 0.25 % serva blue in water, ethanol and acetic acid (4.5, 4.5, 1) for 15 min and destained in the same solution without serva blue to a clear background. Measurements of Rocket apex were made from the top of the sample well to the Rocket apex. Pure proteins in 0.9 % Nacl were used for the calibration of the electroimmunoassay.

Rat albumin secreted into the medium was also determined by immunonephelometry as previously described (10).

The presence of the four proteins mentioned above as well as the content of fibrinogen, α_1-acute-phase protein, α_1-acid glycoprotein and α_1-antitrypsin were also checked by Ouchterlony double immunodiffusion against monospecific antisera in 30-fold concentrated media of 1 and 20 days co-cultures.

RESULTS

As shown in Table I, when mixed with RLEC, adult rat hepatocytes secreted albumin,hemopexin, haptoglobin and α_2-macroglobulin over the 3 weeks period studied. During the first days, the rate of albumin secretion was stimulated, as previously reported (10). In contrast, no important change was observed in the level of hemopexin and haptoglobin over the same period. α_2-macroglobulin was very low in the medium of hepatocyte co-cultures until day 4 ; by days 5-6, its concentration rapidly rose and thereafter remained close to 6 µg/day/10^6 hepatocytes. The levels of secretion observed for the 4 proteins after 6-7 days of co-culture were roughly maintained during the following 2 weeks. The values of the albumin secretion rate measured by electroimmunodiffusion or by immunonephelometry were identical.

In pure culture, secretion of plasma proteins was not measured after 5 days in serum-depleted medium since a large number of hepatocytes detached at this time. A quite different pattern was observed for albumin, haptoglobin and α_2-macroglobulin. While haptoglobin rapidly declined, the amount of albumin secreted increased during the first two days before promptly falling. α_2-macroglobulin was detected in the medium as soon as 24 hr after hepatocyte seeding and its concentration increased up to day 3.

No detectable amounts of albumin, haptoglobin, hemopexin and α_2-macroglobulin were found by electroimmunodiffusion in the medium of pure cultures of RLEC maintained in similar experimental conditions.

The presence of the above mentioned proteins as well as fibrinogen, α_1-acute-phase protein, α_1-acid glycoprotein and α_1-antitrypsin was demonstrated by immunodiffusion in both 1 and 20 day co-cultures (data not shown).

DISCUSSION

When co-cultured with another liver epithelial cell type, adult rat hepatocytes retained the capability to secrete various acute-phase

Table I - Secretion of albumin, haptoglobin, α_2-macroglobulin and hemopexin in the medium of pure and mixed cultures of adult rat hepatocytes.

Days	Albumin	Haptoglobin	α_2-macro-globulin	Hemopexin
CO-CULTURES				
1	-	-	-	-
2	35.4	16.8	0.9	-
3	38.4	10.8	1.2	-
4	46.2	12.0	2.7	13.2
5	55.2	10.2	6.0	6.0
6	49.8	9.9	11.7	7.5
7	42.0	10.5	10.5	8.1
8	51.9	13.5	6.6	10.2
9	46.2	12.6	6.9	8.7
10	48.6	10.8	5.7	6.9
11	46.8	10.2	9.6	6.0
12	42.9	10.2	4.8	7.2
13	43.8	11.1	5.1	9.6
14	41.7	10.8	4.8	7.2
15	38.7	8.1	4.2	5.7
16	36.0	7.5	8.7	5.1
17	-	-	-	-
18	36.0	13.2	7.2	9.6
19	42.6	11.4	8.7	9.9
20	42.9	10.2	6.3	11.1
PURE CULTURES				
1	15.0	6.9	2.0	-
2	20.5	5.5	7.5	-
3	20.8	4.0	6.1	-
4	16.5	1.8	5.2	-
5	13.0	1.5	5.5	-

The values are expressed in µg/day/10^6 hepatocytes and are means of two independent experiments performed in duplicate.
The number of attached hepatocytes was determined by measuring the intracellular lacticodehydrogenase content according to Rubin et al. (30) before addition of RLEC.
(-) not determined.

proteins in addition to albumin (a non acute-phase protein) in serum-free medium for several days.

A number of studies have shown that in conventional culture conditions, adult rat hepatocytes exhibit a dramatic decrease in their secretion rate of plasma proteins within a few days (10, 18-20). Our data on pure hepatocyte cultures maintained for 5 days in serum-free medium fully confirm these conclusions, including the nonparallel decline of the plasma

proteins (4). As an example the secretion of haptoglobin was found to promptly decrease while that of albumin showed a previous temporary increase. Various explanations can be suggested to interpret these data. First, when parenchymal cells are seeded in culture, they still possess a memory of the signals to which they are responding *in vivo*. Second, the isolation process impairs various cellular functions. Third, the culture conditions represent a new and very different environment for the cells, which might be inappropriate for the maintenance of the normal phenotype. A number of factors have been reported to modulate the synthesis of one or more plasma proteins *in vitro* (21). Therefore, the use of isolated or short-term cultured hepatocytes for studying the regulation of plasma protein secretion has evident shortcomings.

In hepatocyte co-cultures, as previously observed for albumin (10), secretion of other plasma proteins remained high and roughly stable for at least 2-3 weeks. This is not related to the participation of RLEC since none of the four proteins was detected by electroimmunodiffusion in the medium of these cells. Moreover, by using the immunoperoxidase technique, we demonstrated that all the hepatocytes but no RLEC contained albumin (22). The early stimulation of albumin production was again found in this study. Such an increase could be related to addition of hydrocortisone to the culture medium since a hormonal dose-response has been previously reported (23). However, the effects of corticosteroids on albumin production remain unclear. Cortisol augments albumin synthesis in patients with hepatocellular diseases (24) and in cultured hepatoma cells (25, 26). In contrast, this protein is not significantly diminished in adrenalectomized rats (5) and is only slightly increased in isolated hepatocytes treated with high doses of corticosteroids (27). α_2-macroglobulin production was also stimulated in co-culture after 4-6 days. This acute-phase protein is actively synthesized in foetal liver but is absent in the serum of normal adult rats (28). The synthesis of this protein in injured animals has been considered as a reversion to some foetal biosynthetic pathway (28). Therefore, it may be asked whether co-cultured hepatocytes partly dedifferentiate during the first days of culture. This seems fairly improbable since proteins normally synthesized in the adult liver, were either stimulated (albumin) or remained unchanged (haptoglobin and hemopexin) in such co-cultures. It is likely that the early increase in α_2-macroglobulin like in albumin, could be attributed to the response of parenchymal cells to their new environment, especially the presence of hydrocortisone in the culture medium. The absence of detectable secretion of α-foetoprotein (not shown), a foetal glycoprotein which is expressed in adult rat hepatocytes when cultured alone for a few days (19), supports the conclusion that parenchymal cells remained differentiated in co-culture.

Four other plasma proteins, fibrinogen, α_1-acute-phase protein, α_1-acid glycoprotein and α_1-antitrypsin assayed by immunodiffusion, were detected on day 20 as on day 1 of co-culture. This indicates that in co-culture, adult rat hepatocytes retained the capability to synthesize a large number, if not all, of plasma proteins normally manufactured *in vivo*.

Finally, hepatocytes co-cultured in serum-free medium can secrete various acute-phase plasma proteins at a relatively constant level for several days. They represent an unique experimental model for studying plasma proteins, especially factors involved in the synthesis of acute-phase proteins (29).

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REFERENCES

1. Jamieson, J.C., Kutryk, M., Woloski B.M.R.N.J., and Kaplan H.A. (1982) Biochem. Med. 28, 176-187.
2. Jamieson, J.C., Morrison, K.E. Molasky, D., and Turchen, B. (1975) Can. J. Biochem. 53, 401-414.
3. Jeejeebhoy, K.N., Ho, J., Greenberg, G.R., Phillips, M.J., Bruce-Robertson, A., and Sodtka, U. (1975) Biochem. J. 146, 141-155.
4. Jeejeebhoy, K.N., Bruce-Robertson, A., Ho, J. Kida, S., and Muller-Eberhard, U. (1976) Can. J. Biochem. 54, 74-78.
5. Crane, L.J., and Miller, D.L. (1977) J. Cell. Biol. 72, 11-25.
6. Hooper, D.C., Steer, C.J., Dinarello, C.A., and Peacock, A.C. (1981) Biochem. Biophys. Acta 653, 118-129.
7. Grieninger, G., Hertzberg, K.M., and Pindyck J. (1978) Proc. Natl. Acad. Sci. (U.S.A.) 75, 5506-5510.
8. Andus, T., Gross, V., Tran-Thi, T.A., Schreiber, G., Nagashima, M., and Heinrich, P.C. (1983) Europ. J. Biochem. 132, 561-571.
9. Guguen-Guillouzo, C., and Guillouzo, A. (1983) Mol. Cell. Biochem. 53/54, 35-56.
10. Guguen-Guillouzo, C., Clément, B., Baffet, G., Beaumont, C., Morel-Chany, E., Glaise D., and Guillouzo, A. (1983) Exp. Cell Res. 143, 47-54.
11. Bernard, N., Lombart, C., and Jayle, M.F. (1976) Biochimie 58, 1429-1431.
12. Guguen, C., Guillouzo, A. Boisnard, M., Le Cam, A., and Bourel, M. (1975) Biol. Gastroenterol. 8, 223-231.
13. Morel-Chany, E., Guillouzo, C., Trincal, G., and Szajnert, M.F. (1978) Europ. J. Cancer 14, 1341-1345.
14. Delers, F., Lombart, C., Domingo, M., and Musquera, S. (1981) Anal. Biochem. 118, 353-357.
15. Gauthier, F., and Mouray, H. (1976) Biochem. J. 159, 661-665.
16. Engler, R., Pointis, J., Rondeau, Y., Judon, C., and Waks, M. (1977) Clin. Chim. Acta 77, 159-165.
17. Laurell, C.B. (1965) Anal. Biochem. 10, 358-361.
18. Haars, L.J., and Pitot, H.C. (1979) J. Biol. Chem. 254, 9401-9407.
19. Sirica, A.E., Richards, W., Tsukada, C., Sattler, C., and Pitot, H.C. (1979) Proc. Natl. Acad. Sci. (U.S.A.) 76, 283-287.
20. Weiner, A.L., and Cousins, R.J. (1983) Biochem. J. 212, 297-304.
21. Crane, L.J., and Miller, D.L. (1983) Mol. Cell Biochem. 53/54, 89-109.
22. Guillouzo, A., Beaumont, C., Le Rumeur, E., Rissel, M., Latinier, M.F., Guguen-Guillouzo, C., and Bourel, M. (1982) Biol. Cell 43, 163-172.

23. Baffet, G., Clément, B., Glaise, D., Guillouzo, A., and Guguen-Guillouzo, C. (1982) Biochem. Biophys. Res. Comm. 109, 507-512.
24. Cain, G.D., Mayer, G. and Jones, E.A. (1970) J. Clin. Invest. 49, 2198-2204.
25. Brown, P.C. and Papaconstantinou, J. (1979) J. Biol. Chem. 254, 9379-9384.
26. Chou, J.Y., Ito, F., Evans, G., Chiu, J.F. and Feldmann, M. (1982) J. Cell Biol. 93, 314-317.
27. Weigand, K., Wernze, H., and Falge, C. (1977) Biochem. Biophys. Res. Comm. 75, 102-110.
28. Koj, A. (1974) In Structure and Function of plasma proteins (Allison A.C. ed.), pp 78-125, Vol. 1 Plenum Press (London).
29. Rupp, G., and Fuller, G.M. (1979) Exp. Cell Res. 118, 23-30.
30. Rubin, K., Oldberg, A., Hook, M. and Obrink, B. (1978) Exp. Cell Res. 117, 165-177.