Long-term biosynthesis of complement component C3 and α -1 acid glycoprotein by adult rat hepatocytes in a co-culture system with an epithelial liver cell-type

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We used a system of co-culture of adult rat hepatocytes with another epithelial cell type from rat liver to study the synthesis of two acute-phase reactants, α -1 acid glycoprotein (α 1AGP) and the third component of complement (C3), and we have obtained long-term secretion of these two proteins. After a period of adaptation corresponding to the first 2–4 days of the co-culture, hepatocytes secreted C3 and α 1AGP for at least 2 weeks at a mean level higher than that observed in the first days of a pure culture of hepatocytes. When pulse-chase analysis was performed on day 6 of co-culture, kinetics of synthesis of α 1AGP and C3 were the same as those observed on day 1 of a conventional culture of pure hepatocytes. Furthermore, intracellular and extracellular α 1AGP had M_r values respectively of 39000 and of 42000–52000, identical with those observed in pure cultures of hepatocytes. Similarly, the molecular size and subunit structures of C3 were the same in co-culture and in cultures, indicating an identical processing of this protein. C3 produced in co-culture was also haemolytically active. Therefore, the system of adult hepatocytes co-cultured with this liver epithelial cell provides a physiological system *in vitro* which permits long-term synthesis of the two acute-phase reactants C3 and α 1AGP. This model opens the possibility to study the modulation of the synthesis of these two proteins during a long period by inflammatory agents or by hormones.

INTRODUCTION

Hepatoma cells of rat (Strunk *et al.*, 1975) and human (Morris *et al.*, 1982a,b) origin or primary cultures of guinea-pig hepatocytes (Ramadori *et al.*, 1984) have been used to study the synthesis of C3 complement component. Liver cell suspensions (Jeejeebhoy *et al.*, 1975; Crane & Miller, 1977; Hooper *et al.*, 1981), liver slices (Peters, 1973), human (Carlson *et al.*, 1984) and mouse and rat (Bernhard *et al.*, 1973; Ledford & Davis, 1983; Baumann & Held, 1981) hepatoma cells, chick embryo hepatocytes (Grieninger & Granick, 1978) and adult rat hepatocytes (Gross *et al.*, 1982; Andus *et al.*, 1983) in primary monolayer culture have been also used to study the hepatic synthesis of albumin and several plasma proteins which include those known as acute-phase reactants (Koj, 1974).

However, after a few days in culture, adult hepatocytes do not retain their differentiated state and demonstrate phenotypic changes characteristic of fetal liver parenchymal cells (Guguen-Guillouzo & Guillouzo, 1983). Moreover, these cultures pose the problem of being too short-lived and do not allow experiments of several days duration. On the other hand, all hepatoma-derived cell lines are not able to synthesize complement components as a whole (Strunk *et al.*, 1975), and conflicting results have been also obtained as regards the biosynthesis of C3 after adding glucocorticoids to different establishment rat hepatoma cell lines (Strunk *et al.*, 1975; Fey *et al.*, 1983). Recently, a method has been devised which permitted

normal adult rat hepatocytes to maintain, *in vitro*, their capability of producing a high level of albumin for several weeks (Guguen-Guillouzo *et al.*, 1983). This method is based on the association of rat parenchymal liver cells with an undifferentiated epithelial cell line derived from rat liver.

The objectives of the present study were (1) to obtain evidence that C3 and α 1AGP were secreted during the period of the co-culture, (2) to determine whether the C3 produced was haemolytically active, and (3) to establish the precursor-product relationship between the cellular and the secreted forms of these proteins in this model.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats were obtained from IFFA-CREDO (France) and were used at 10–15 weeks of age as a source of hepatocytes.

Cell cultures

Pure cultures of rat hepatocytes were prepared essentially as described (Seglen, 1973). The animals were anaesthetized with Nembutal (0.1 ml/100 g body wt.) injected intraperitoneally, and the abdomen was opened to cannulate the vena porta and to section the vena cava. The liver was washed *in situ* at 37 °C with a solution, pH 7.6, containing 0.4 g of KCl, 8 g of NaCl, 2.4 g of Hepes and 0.2 g of $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$ /litre. Thereafter,

the organ was perfused with the same buffer supplemented with 0.025% collagenase (Worthington, Freehold, NJ, U.S.A.) and 5 mM-CaCl₂. Then the liver was removed and the cell suspension was filtered and decanted for 30 min in William's E medium (Gibco) supplemented with albumin (1 g/l), insulin (10 mg/l), and Hepes (2.4 g/l) at pH 7.4. After washing and centrifugation at 800 rev./min, the cell suspension showed a viability of 85–90% as determined by the Trypan Blue exclusion test. After resuspension in the medium as above, containing 10% heat-inactivated fetal calf serum and antibiotics, 2×10^6 cells in 4 ml were plated and incubated at 37 °C in an incubator under humidified CO₂/air (1:19) for 4 h. At this time, unattached cells were removed and the flasks replenished with 3 ml of fresh medium supplemented with dexamethasone (1 μ M). The survival rate of adult hepatocytes is significantly increased by corticosteroids, with maintenance of the polygonal epithelium morphology (Guguen *et al.*, 1975; Laishes & Williams, 1976). Confluent monolayers were formed after an overnight incubation. Supernatants were harvested at different times and stored at –70 °C before testing.

Mixed cultures of adult rat hepatocytes were performed as described (Guguen-Guillouzo *et al.*, 1983). Briefly, the rat epithelial cells were obtained from the liver of a normal 10-day-old Fisher rat (Morel-Chany *et al.*, 1978) and were used at the early passages. Hepatocytes were isolated by collagenase perfusion; 2×10^6 hepatocytes were suspended in 3 ml of William's E medium containing 1 g of albumin/l, 5 mg of insulin/l and 10% fetal calf serum, and then were plated in 25 cm² culture flasks. Within 4 h, about 70% of the seeded hepatocytes attached to the plastic. At this time, the medium and the dead hepatocytes were removed and 4×10^6 epithelial cells were added in 3 ml of fresh medium supplemented with 1 μ M-dexamethasone. Corticosteroids appear to be essential for long-term survival of active albumin-secreting hepatocytes co-cultured with epithelial cells. Their effects are dose-dependent and, in their absence, rat hepatocytes do not survive for more than 2 weeks (Baffet *et al.*, 1982). The flasks were incubated as above and the medium was renewed every day. Supernatants were harvested at different times and stored at –30 °C before testing.

Measurement of DNA levels in monolayers

Cells were removed by scraping after lysis by addition of 0.4% Triton X-100 and incubation for 30 min at 4 °C. Samples were stored in polypropylene tubes at –70 °C. Aliquots (1 ml) were assayed for DNA (Adams, 1981). We found that 1×10^6 rat hepatocytes contain approx. 10 μ g of DNA.

Purification of rat plasma C3 and α 1AGP

Rat C3 was purified from plasma by a four step procedure including precipitation with 4–12% poly(ethylene glycol), a DEAE-Sephacel ion-exchange chromatography, an affinity chromatography on phenyl-Sepharose, and a gel-filtration on Sephacryl S-200. Rat plasma, containing 0.02 M-EDTA, 0.05 M-6-aminohexanoic acid and 0.01 M-benzamidine, pH 7.2, was precipitated as described for human C3 (Al Salihi *et al.*, 1982). After the two steps following, namely the DEAE-Sephacel and phenyl-Sepharose chromatographies, the preparation still contained α_2 -macroglobulin, which was eliminated by gel filtration on Sephacryl S-200 (100 cm $\times$ 2.6 cm)

equilibrated with 0.15 M-NaCl, 0.02 M-EDTA, 0.05 M-6-aminohexanoic acid and 0.001 M-benzamidine. Elution of C3 in each chromatographic experiment was monitored by its haemolytic activity determined by a one-step method using a human serum depleted of C3 (R3 reagent) prepared by immunoadsorption (Fontaine *et al.*, 1980). Briefly, 0.5 ml of erythrocyte-antibody (10^8 cells/ml) was incubated with 0.5 ml of R3 diluted 20-fold and with 0.5 ml of the source of C3 during 1 h at 37 °C. The reaction was stopped by adding 3.5 ml of chilled PBS. Tubes were centrifuged at 2500 rev./min and the A_{414} of the supernatants was measured. Results were expressed as the number of effective molecules/mg of protein according to the formula $Z = -\ln(1 - y)$, where y is the percentage of haemolysis and Z represents the number of haemolytic sites per cell.

Rat α 1AGP was extracted from plasma obtained 48 h after subcutaneous injection of turpentine oil (0.6 ml/100 g body wt.) and was purified essentially as described by Nicollet *et al.* (1981) for human α 1AGP. The homogeneity of the protein was established by SDS/polyacrylamide-gel electrophoresis and by immunoelectrophoresis using rabbit antisera against α 1AGP and against the normal rat serum prepared in our laboratory.

Assay for rat C3 and α 1AGP

Rat plasma and supernatants of pure and mixed cultures of rat hepatocytes were assayed by an e.l.i.s.a. Monospecific antisera to rat C3 and to α 1AGP were raised in a rabbit, and specific antibodies were obtained by affinity chromatography on a CNBr-Sepharose 4B column for rat C3 and by affinity chromatography on a glutaraldehyde-activated acrylamide/agarose column (ACA-22; IBF, France) for rat α 1AGP. Alkaline phosphatase (Boehringer Mannheim, Germany) was coupled to these antibodies with glutaraldehyde in a two-step procedure (Avrameas & Ternynck, 1971). The polystyrene micro-titration plates from Costar (Cambridge, MA, U.S.A.) were coated with 250 μ l/well of a 10 μ g/ml solution of monospecific C3 or α 1AGP antiserum in 0.1 M-bicarbonate buffer, pH 9.0, and incubated overnight at 37 °C. After three washes with PBS, the plates were filled with PBS containing 0.5% bovine serum albumin and incubated at 37 °C for 2 h, then washed again with PBS, pH 7.2, containing 0.05% Tween 20. Then samples were added at different dilutions in PBS containing 5% bovine serum albumin. After incubation overnight at 37 °C, the plates were washed six times with PBS containing 0.005% Tween 20. Each well was then filled with 250 μ l of enzyme-labelled specific antibody for a further incubation for 4 h at 37 °C, followed by six washes with PBS/Tween and two washes with distilled water. Bound conjugate was revealed using 250 μ l/well of a solution of *p*-nitrophenol phosphate (1 mg/ml) in 0.1 M-bicarbonate buffer, pH 9.8, containing 1 mM-MgCl₂ and 1 M-diethanolamine. The A_{405} was then read at various times by a semi-automated spectrophotometer (Bio-tek Instruments). For the standard curves, each plate included in duplicate 250 μ l of standard rat α 1AGP or C3 at six different concentrations. The e.l.i.s.a. gave standard curves which were approximately linear from 3 μ g/l to 100 μ g/l. The specificity of the assay was tested by measuring a number of negative controls, including sera depleted of α 1AGP and C3 respectively, which did not exhibit significant absorbance as compared with the blanks.

Fig. 1. Phase-contrast photomicrograph ($\times 200$), at 8 days, of adult rat hepatocytes co-cultured with rat liver epithelial cells

H, hepatocytes; E, liver epithelial cells.

Pulse-chase labelling of intracellular and extracellular proteins

Growth medium was removed from rat hepatocyte primary cultures and co-cultures and was replaced with 2 ml of methionine-deficient medium. After a 1 h equilibration period, 200 μ Ci of L-[35 S]methionine (Amersham; 1000 Ci/mmol) was added to each flask. Incorporation was allowed to proceed for 2 h. The medium was then removed, cultures were washed and 2 ml of medium containing 300 mg of non-radioactive methionine/l was added. Different chase times were performed. At the end of each chase period, the medium was removed: the cells were harvested, washed and lysed in 2 ml of 10 mM- Na_2 EDTA, pH 7.2, containing 1% Triton X-100, 0.5% sodium deoxycholate, 2 mM-phenylmethanesulphonyl fluoride and 0.15 M-NaCl, and centrifuged for 10 min at 12000 g .

Immunoprecipitation

For the immunoprecipitation, 250 μ l of sample was added to 45 μ l of protein A-Sepharose and to 10 μ l of specific antiserum (anti-C3 or anti- α 1AGP). After an incubation at 0 $^{\circ}\text{C}$ overnight, the antigen-antibody complexes bound to protein A-Sepharose were washed five times with buffer A [50 mM-Tris/HCl (pH 7.2)/0.15 M-NaCl/0.1% Triton X-100/0.1% sodium deoxycholate] and five times with buffer B (buffer A containing 1 M-NaCl). Elution was achieved by incubation with 1 M-Tris/HCl (pH 7.8)/0.4 M-dithiothreitol/10% SDS/10% glycerol at 95 $^{\circ}\text{C}$ for 5 min. The eluted proteins were analysed by electrophoresis on to a 3% SDS/polyacrylamide stacking gel, followed by separation on a 10% SDS/polyacrylamide gel with a discontinuous buffer (Laemmli, 1970). The gels were stained with Coomassie Brilliant Blue, destained and subjected to fluorography.

RESULTS

Production of C3 and α 1AGP by epithelial cells

Supernatants of confluent monolayers of 2.5×10^6 pure epithelial cells were harvested after 48 h of culture. The mean α 1AGP value of three separate experiments was 15 ng/ml, i.e. 0.3 ng/h per 10^6 cells, and the mean C3 value was 19 ng/ml, i.e. 0.4 ng/h per 10^6 cells.

Production of C3 and α 1AGP in the co-culture

As observed in Fig. 1, a phase-contrast micrograph of adult rat hepatocytes co-cultured with liver epithelial cells showed epithelial cells in close contact with hepatocytes which exhibited a morphology consisting of a granular cytoplasm and one or two nuclei. In cultures of pure hepatocytes, C3 and α 1AGP secretion showed a dramatic decrease after 2 days and was not detectable after 5 days (Table 1). By contrast, quite different patterns of C3 and α 1AGP production were observed when hepatocytes were co-cultured with epithelial cells. Supernatants of co-cultures were measured daily over a 10-day period and were still harvested and measured at day 20 for C3 and at day 12 for α 1AGP. Levels of secretion observed for C3 were low until day 2 and declined until day 4 for α 1AGP. Then an increase in α 1AGP (day 5) and C3 (day 3) occurred and was maintained at relatively high levels with slight variations during the period studied. At that time, levels of secretion always remained higher than during the first days of co-culture. For C3, the mean of the values measured during the period of 9 days after the first 2 days of co-culture was 39 ng/h per 10^6 cells. For α 1AGP, the mean of the values during the 7 days after the first 4 days of co-culture was 75 ng/h per 10^6 cells (values expressed as mean of three experiments; Table 1). These values were respectively 130-fold higher for C3 and more than 185-fold higher for α 1AGP in the co-culture as compared with the amounts detected in the pure culture of epithelial cells.

Table 1. Secretion of α 1AGP and C3 in the medium of hepatocytes in pure cultures and of hepatocytes in co-cultures

Supernatants were harvested from monolayers of pure cultures and co-cultures which were obtained as described in the Materials and methods section. Media were harvested and renewed daily, and secretion was measured by e.l.i.s.a. Values, expressed in ng/h per 10^6 cells, are the average of triplicate experiments. Abbreviation used: ND, not determined.

Time (days)	Secretion (ng/h per 10^6 cells) of:			
	α 1AGP		C3	
	Hepatocytes in pure cultures	Hepatocytes in co-cultures	Hepatocytes in pure cultures	Hepatocytes in co-cultures
1	76	60	33	19
2	78	58.5	16	19
3	32	53	11	47
4	20	28	8.5	43
5	12	86	2	36
6	6	91	2	31
7	4	59	2	36
8	4	83	2	40
9	ND	64	2	39
10	ND	76	ND	40
20	ND	74	ND	40

Stability of proteins in media of epithelial cells

In order to determine whether C3 could be cleaved by products released from epithelial cells, ^{125}I -C3 (4×10^6 c.p.m.) was added to 3 ml of the culture medium of these cells (3×10^6 cells/flask). Aliquots (150 μl) were harvested at 15, 30, 60, 90 and 120 min and analysed by SDS/polyacrylamide-gel electrophoresis in reducing conditions followed by autoradiography. Only the two bands corresponding to the α -chain (M_r 115000) and β -chain (M_r 68000) were seen as compared with the α - and β -chains of ^{125}I -C3 used as marker (results not shown), thus suggesting that no proteolytic activity was present in media of epithelial cells.

Production of functional C3 complement component activity

The method of purification outlined in the Materials and methods section allowed the recovery of 42% of the C3 present in rat plasma. This C3 was haemolytically active and was free of C5 and factor H. The M_r of rat C3 was equivalent to that of human C3 (185000) and rat C3 was dissociated on reduction into two polypeptide chains of M_r 110000 and 75000 after SDS/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969). Injection into rabbits of our preparation elicited the production of a specific antibody that permitted us to measure the plasma level of rat C3. This concentration was 1.60 ± 0.23 mg/ml (30 rats), giving a specific haemolytic activity of 2.9×10^{11} effective molecules/mg. Supernatants in triplicate of co-cultures were measured at day 6 and gave a specific haemolytic activity of 1.84×10^{11} effective molecules/mg.

Precursor-product relationship between intracellular and extracellular α 1AGP and intracellular and extracellular C3

Hepatocytes were pulse-labelled with L- ^{35}S methionine for 2 h on day 1 after plating of hepatocytes alone, and on day 6 after plating of hepatocytes co-cultured with

epithelial cells. Hepatocytes were then chased in medium containing non-radioactive methionine for various periods, as indicated in Figs. 2 and 3. A continuous decrease of radioactivity of the intracellular content was accompanied by an increase of radioactivity in the secreted medium. As seen in Figs. 2 and 3, the rate of secretion of [^{35}S]methionine-labelled C3 and α 1AGP was maximum after 60 min of chase. Intracellular α 1AGP had an apparent M_r of 39000, much less than the extracellular protein, which exhibited a large difference of M_r ranging from 42000 to 52000. Pro-C3 (M_r 185000), C3 α -chain (M_r 115000) and C3 β -chain (M_r 68000), together with the autocatalytic forms of α -chain of C3 (M_r 76000 and 44000) were seen in the medium and in the cellular lysates of cultures and co-cultures; these latter forms were barely visible in the medium of the co-cultures. When unlabelled C3 (10 μg) was added into the immunoprecipitation assay which was followed by electrophoresis, bands corresponding to pro-C3, α -chain and β -chain were no longer apparent except, in cell lysates of the co-culture, for a protein with a mobility slightly less than the myosin marker and probably representing fibronectin.

DISCUSSION

We were able to obtain a long-term maintenance of the synthesis of C3 and α 1AGP by using a co-culture system associating adult rat hepatocytes and another liver epithelial cell type, as recently described (Guguen-Guillouzo *et al.*, 1983). The results of our study are consistent with this previous observation which showed that synthesis of albumin could be obtained during a period far beyond that observed in conventional pure hepatocyte monolayers. No C3 and α 1AGP were detected in the medium of pure liver epithelial cells. We have also previously shown that no albumin was found to be secreted in the medium of cultures of epithelial cells, since this protein was observed in all or almost all of the hepatocytes, but never in epithelial cells, by using the

Fig. 2. Pulse-chase analysis of C3 in hepatocyte cultures (a) and in co-cultures (b) from an adult male rat on day 1 after plating of pure cultures, and on day 6 after plating of the co-cultures

Cultures were pulse-labelled with 200 μ Ci of [35 S]methionine for 2 h after 1 h equilibration period of the culture in a methionine-deficient medium. The radioactive medium was removed and cultures were chased in medium containing non-radioactive methionine for various periods of time, as indicated. After the chase, medium was removed, cells were lysed and C3 was precipitated from cell lysates and culture medium as indicated in the Materials and methods section. Autoradiograms of SDS/polyacrylamide gels under reducing conditions are shown. Lanes S and L contain respectively the culture supernatants and the cell lysates. On the right (b), M_r markers are, from top to bottom: myosin (200000), phosphorylase *b* (92500), albumin (68000), ovalbumin (43000) and carbonic anhydrase (30000).

Fig. 3. Pulse-chase analysis of α 1AGP in hepatocyte cultures (a) and in co-cultures (b) from an adult male rat on day 1 after plating of pure cultures, and on day 6 after plating of the co-cultures

For conditions, see the legend to Fig. 2.

immunoperoxidase technique. The stimulation of albumin production was only due to a close contact between hepatocytes and epithelial cells. The establishment of cell-cell contacts between hepatocytes and liver epithelial cells is essential for the maintenance of a high rate of transcription of the liver-specific genes (Fraslin *et al.*, 1985), since neither a conditioned medium nor an epithelial cell extract could induce the same response (Guguen-Guillouzo *et al.*, 1983). The results reported here during the pulse-chase experiments provide also formal proof that C3 and α 1AGP were synthesized by the hepatocyte cells in co-cultures and were not a release of a material associated with the cells. Furthermore, the kinetics of synthesis were not different from those observed in pure cultures of hepatocytes, since the time of appearance of newly synthesized α 1AGP and C3 in the media was the same in the pure cultures of hepatocytes on day 1 and in co-cultures on day 6. We have, however, observed that the β -chain of C3 was less detectable in media samples from co-cultured cells as compared with that observed in pure cultures. Nevertheless, this was apparently not due to a proteolytic effect. The strong band observed on SDS/polyacrylamide-gel electrophoresis of media of co-cultures has an M_r identical with that of fibronectin and is not complement protein related. As a matter of fact, it has been shown (Guguen-Guillouzo *et al.*, 1983) that, after establishment of cell-cell contacts, rapid production of an extracellular material occurs, which is located between the two cell types and which contains fibronectin and various collagen types. A very large difference between the M_r of secreted and cellular forms was found for α 1AGP both in pure hepatocyte cells and in co-cultured cells. Intra- and extra-cellular forms of this glycoprotein differ greatly in carbohydrate structures; the cellular form corresponds to a polypeptide with a high-mannose structure and the secreted form has a very high carbohydrate content (41% of total weight) with a complex oligosaccharide structure. This probably explains the great heterogeneity in M_r , as also recently observed in primary cultures of hepatocytes (Andus *et al.*, 1983) and in rat hepatoma cells (Vannice *et al.*, 1983). The observation reported here concerning the secretion of haemolytically active C3 may be explained by the fact that pro-C3, the single-chain precursor molecule of C3, present in the cellular lysate, was appropriately processed into the mature haemolytic forms of C3. Again, the appearance of the two autolytic fragments of M_r 76000 and 42000 of the α -polypeptide chain, which appear under certain conditions used to prepare the protein for SDS/polyacrylamide-gel electrophoresis, indicates the presence of native C3, since these fragments occur only with haemolytically active C3 and are not observed with inactivated C3 or with C3b (Sim & Sim, 1981). Finally, the decrease in haemolytic activity (about 30%) may correspond to a spontaneous inactivation which is usually observed and is probably due to the hydrolysis of the thioester bond (Pangburn & Müller-Eberhard, 1980).

Most of the studies, for C3, C4 and α 1AGP, have dealt exclusively with their biosynthesis in primary hepatocyte cultures during periods which do not exceed 24 or 48 h (Ramadori *et al.*, 1984; Gross *et al.*, 1984; Rosa & Shreffler, 1983). Complement biosynthesis has been also studied in the human hepatoma-derived cell line HepG2 (Morris *et al.*, 1982b). However, even if cultured cell lines derived from hepatomas can continue to express functions which are characteristic of the normal liver,

they can differ widely one from another in the spectrum of functions expressed. For example, HepG2 synthesizes and secretes several functional complement factors but fails to produce detectable C1q, C7 and C9.

In summary, the system of adult hepatocytes co-cultured with liver epithelial cells permits a long-term synthesis of the two acute-phase reactants C3 and α 1AGP. This opens the analysis for the regulation of the synthesis of these two proteins *in vitro* in a physiological model.

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