EXPRESSION OF γ-GLUTAMYL TRANSPEPTIDASE IN IAR 2 CELLS CULTURED ON ADHESIVE AND NONADHESIVE SUBSTRATES

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Interest in the study of γ-glutamyl transpeptidase (GGT; EC 2.3.2.2) is due to the fact that this enzyme can act as an early marker of hepatocyte transformation. In normal rats high GGT activity characterizes hepatocytes of late embryos and cannot be detected histochemically in hepatocytes of adult rats [4]. However, in the early stages of carcinogenesis GGT activity rises sharply in the transformed cells. Moreover, GGT activity correlates closely with the tumorigenicity of the epithelial cells of the liver during their transformation in tissue culture [6]. GGT activity, detected histochemically, has been found to be distributed unevenly in cultures of epithelial cells transformed *in vitro* [1]. Cells of tumorigenic epithelial lines from rat liver have also been shown to differ always from their nontumorigenic precursors by being less able to spread out on a substrate [3]. It might be supposed that an increase in GGT activity in tumorigenic cells is the result of the weaker spreading power of the cells and the formation of local stratified areas. To test this hypothesis the effect of impairment of spreading and of cell aggregate formation on GGT activity was studied in nontumorigenic rat liver epithelial cells.

EXPERIMENTAL METHOD

Liver epithelial cell line IAR 2 was obtained in 1973 from a primary culture of BD4 rat hepatocytes [7]. The cells were grown in Williams E medium (Flow Laboratories, England), with the addition of 10% embryonic calf serum (Gibco, England) and 100 U/ml of monomycin. A suspension with a cell concentration of $2 \cdot 10^4$/ml of medium was used for subculture. The nonadhesive substrate was obtained by diluting a concentrated solution of Hydron [poly-(2-hydroxyethylmethacrylate)] (Hydron Laboratories, USA) in alcohol in the ratio of 1:250, according to the technique in [5].

Rutenberg's method [10] was used for histochemical staining. Before staining the cells were fixed with 10% formaline for 40 min. After specific staining the cells were counterstained with hematoxylin and mounted in buffered glycerin.

Biochemical activity of GGT was determined after extraction of the cells with a 1% solution of Triton X-100 in 5 mM Na-phosphate buffer, pH 7.2, by the method in [9]. Incubation was carried out for 1 h at room temperature. The protein concentration was determined by a modified Lowry's method [2]. To determine biochemical activity the solubilizates from three Petri dishes (diameter 5 cm) were pooled and concentrated by ultrafiltration on a PM 10 membrane (Amicon, USA). The determination was done in two parallel tests. To inhibit protein biosynthesis a 0.5 mM solution of cycloheximide (Serva, West Germany) in culture medium was used. The intensity of biosynthesis was determined from the incorporation of ^{14}C-glycine, present in the culture medium in a concentration of 10 μCi/ml. Cycloheximide was added for 24 h. After removal of the cycloheximide the cells were washed twice with PBS, twice with cold methanol, and twice with cold 5% TCA. The cells were then dissolved in 0.5 ml of 0.3 M NaOH and counted in 10 ml of dioxan scintillator. During culture on Hydron protein biosynthesis was inhibited by 70%, and on glass by 61.4%.

EXPERIMENTAL RESULTS

IAR 2 cells adherent to Hydron spread out extremely slowly. Even after culture for 24 h they formed "islets" of very poorly spread out cells, and in some of them GGT was found by

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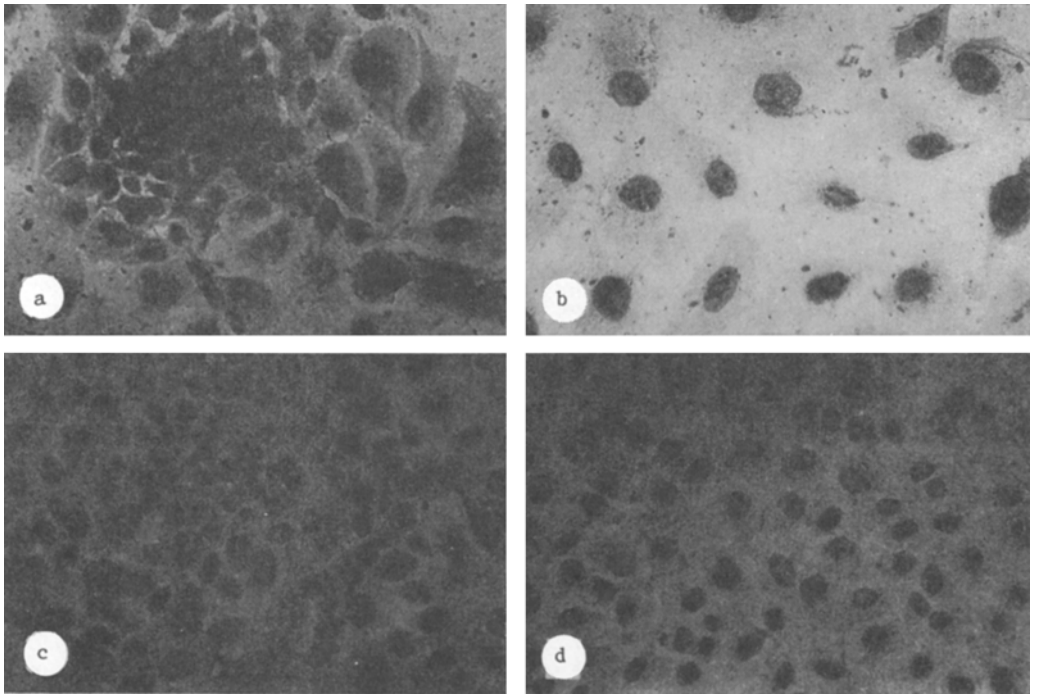

Fig. 1. Expression of GGT activity during culture of IAR 2 cells on Hydron and on glass: a) culture for 1 day on Hydron, b) culture for 1 day on glass, c) culture for 3 days on Hydron, d) culture for 3 days on glass. Histochemical staining for GGT with counterstaining with hematoxylin. 450×.

histochemical staining (Fig. 1a). During culture of these same cells on glass they were distributed uniformly over the substrate, they spread out well, and they contained no GGT (Fig. 1b). After culture for 48 and 72 h on Hydron the cell "islets" gradually became flatter because of increasing spreading, which began from the edges of the "islets." Under these circumstances the intensity of their histochemical staining gradually weakened (Fig. 1c, d). The abrupt slowing of spreading out on Hydron was reflected by delay of DNA synthesis by the cells when cultured on this substrate (Table 1).

To confirm the histochemical data experiments were carried out to measure GGT activity in extracts from cells cultured on both substrates. Activity was measured in the original layer of cells, in their suspension, and after culture for 2, 3, 4, 24, 48, and 72 h. The absolute values of specific activity of GGT in the cells were measured from experiment to experiment and showed a definite tendency to increase with an increase in the number of subcultures (Table 2). It will be clear from Table 2 that 24-48 h after subculture the cells cultured on Hydron exhibited 2-3 times higher GGT activity than that observed in cells cultured on the ordinary substrate. GGT activity in the cells cultured on Hydron subsequently decreased. The results of the biochemical measurements thus confirmed those obtained by the histochemical method: In poorly spreading "islets" of nontumorigenic IAR 2 cells GGT activity rose sharply, and as normalization of the morphology of the cell layer cultured on Hydron took place, the difference in GGT expression was reduced. Cycloheximide, in a dose inhibiting protein biosynthesis by 70%, depressed the peak of GGT activity after culture on Hydron for 24 h. This is evidence that the increase in GGT activity was linked at least partially with protein biosynthesis. The greatest difference in the value of GGT activity in cells cultured on Hydron and on glass, after culture for 1-2 days, coincided with the maximal morphological differences observed at this time.

Yet another important difference in GGT expression in cells cultured on Hydron and on glass was observed 2 h after seeding of the cells on the corresponding substrate. It will be clear from Table 2 that GGT activity during the first 2 h was lower in cells transplanted onto glass than in the suspension, whereas in cells transplanted onto Hydron it was 1.5 times higher. Possibly the short-term increase in GGT activity during the first hours after fixation to the nonadhesive substrate can be attributed to the fact that the cells were left for a long time in a rounded state, since the spreading process was impeded. GGT is known to be

TABLE 1. Incorporation of ^{3}H-Thymidine by IAR 2 Cells During Culture on Hydron and on Glass (in CPS/mg protein)

Time, days	Hydron	Glass
1	2 860	10 100
2	5 000	21 000
3	18 200	18 000
4	9 600	9 500

Legend. Cells cultured in medium containing ^{3}H-thymidine (10 μCi/ml) for 18 h.

TABLE 2. Expression of GGT Activity (in nanomoles p-nitroaniline/mg protein/h) during Culture of IAR 2 Cells on Hydron and on Glass

Expt. No.	Monolayer	Suspension	Time, h					
			2	3	4	21	48	72
1H	—	—	52	26	23	47	56	—
1G			9	9	9	9	48	—
2H	—	—	140	160	100	110	70	100
2G			10	45	10	48	50	90
3H	80	160	—	—	—	910	370	120
3G			—	—	—	460	380	24
4H	270	270	280	240	200	200	900	180
4G			65	18	200	65	430	100
5H	250	250	400	350	180	970	200	300
5G			150	170	300	360	350	320
6H	—	—	—	—	—	330	680	250
6G			—	—	—	280	320	200

Legend. H) Cells cultured on Hydron, G) cells cultured on glass.

TABLE 3. Effect of Versene on Expression of GGT (in nanomoles p-nitroaniline/mg protein/h) in IAR 2 Cells Cultured on Hydron and on Glass

Substrate	Cuture for 3 days	Culture for 3 days+ versene	2 h after washing off	3 h after washing off	4 h after washing off
Hydron	300	570	350	250	200
Glass	310	300	220	260	230

localized mainly on the plasma membranes of cells; moreover, the active center of this enzyme is located on the cell surface [8, 11]. The prolonged and substantial change in the curvature of the cell surface may contribute to the increase in GGT activity. This hypothesis is confirmed by the fact that rounding of the cells with versene leads to a marked increase in GGT activity after only 20 min (at the time the versene was washed off). GGT activity in cells cultured on glass, 2 h after washing off the versene, was about one-third lower than in cells cultured on Hydron (Table 3).

Whatever the molecular mechanisms of the changes in GGT activity, it is clear from the present investigation that impairment of the normal processes of spreading and formation of cell junctions leads to increased GGT expression by these cells. The results confirm the writers' view that an increase in GGT activity during transformation of epithelial cells in tissue culture is the result of lasting impairment of their ability to spread. Accordingly, it is

important to postulate that reexpression of GGT in proliferating precancerous foci and tumors of the liver is the result of a change in junctions between the cells and the extracellular matrix.

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INHIBITORS OF SEROTONIN REUPTAKE AND SPECIFIC IMIPRAMINE BINDING IN HUMAN BLOOD PLASMA

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Many investigators have recently shown interest in the mechanism of the pharmacologic action of tricyclic antidepressants (TAD) in general and of imipramine (IMI) in particular. High-affinity binding sites of IMI and other TAD have been found in parts of the brain [6, 9, 10] and in platelets [11] of man and animals, of a decrease in the number of these sites in endogenous depression [2, 11, 12, 15], and of normalization of IMI binding in patients responding positively to treatment with TAD [8, 13], point to possible interaction between TAD and high-affinity binding sites of IMI, determining their antidepressant activity. The presence of specific IMI binding sites in brain tissue and at the periphery also suggests the existence of an endogenous imipramine-like ligand (or ligands) in man and animals, which may participate in the developmentof affective disorders in man [5, 7, 12].

In 1983 a low-molecular-weight endogenous ligand of IMI binding sites, capable of inhibiting binding of ^{3}H-IMI with its specific binding sites and serotonin (5-hydroxytryptamine, 5-HT) reuptake by rat brain synaptosomes [3], was discovered in brain tissue.

This paper describes a method of extraction of endogenous inhibitors of specific IMI binding and of 5-HT reuptake, from human blood plasma and the heterogeneity of these compounds is demonstrated.

EXPERIMENTAL METHOD

Blood (600 ml) from healthy donors (aged 25-30 years) was collected in plastic tubes containing anticoagulants (0.25 ml of 0.15 M EDTA to 10 ml of blood) and centrifuged at 200g for 20 min at room temperature. Platelet-enriched plasma was separated and centrifuged (10,000g, 10 min, 4°C). The plasma was collected and used for extraction, as described below. The platelet residue was washed twice (10,000g, 10 min, 4°C) in 66 ml of medium containing 50 mM Tris-HCl, 20 mM EDTA, 150 mM NaCl, pH 7.5. The residue was treated with an equal quan-

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