Different Expression of Esterase Variants in Rat Hepatic and Hepatoma-Derived Cell Lines Detected by Electrophoresis

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Esterases in nine rat hepatic and hepatoma-derived cell lines and normal rat liver homogenate were detected by polyacrylamide gel electrophoresis coupled with active staining with α-naphthyl acetate or butyrate as a substrate. The esterase band patterns of the non-cancerous and oncogene-transformed cell lines were alike, but different from those of hepatoma cell lines and normal rat liver homogenate. The former groups of cells might have completely lost the characteristics of rat liver parenchymal cells, or else they might have their origin at cells other than liver parenchyma. The esterase patterns of the hepatoma cell lines (*e.g.*, McA-RH7777) and the normal rat liver highly resembled with each other, exemplifying the slight biochemical deviation of cancer from normal cells. However, two-dimensional electrophoretogram for the McA-RH7777 cell line showed a prominent esterase spot (p*I* 6.0-M_r 110 kDa) that was lacking in the normal liver. This result indicates that there is invariably some change in esterase expression between the cancer cells and the normal liver cells.

Introduction

Recently, we have found that several chiral esters are stereoselectively hydrolyzed by cultured cancer cells and their stereoselectivity is often different or even reversed from that of the corresponding normal tissue, leading to the possibility that cell-specific activation (*i.e.*, cancer targeting) of drugs may be controlled by chirality of the acyl group temporarily protecting drugs to inactive form (Ogawa *et al.*, 1994; Yamazaki *et al.*, 1995). However, the stereoselectivity is greatly dependent on the cancer cell lines, for example, as our latest study has shown, non-cancerous and oncogene-transformed rat hepatic cell lines preferentially hydrolyzed the (*R*)-enantiomer of ethyl (±)-α-methoxyphenylacetate, whereas rat hepatoma cell lines had weak preference to the counter enantiomer (Kageyama *et al.*, 1995). The latter stereoselectivity resembled that of normal rat liver homogenate (Yamazaki *et al.*, 1995). For design of anticancer prodrugs, it is very important to learn how differently the esterase variants are expressed in the different cell lines. The relation between change of esterase isoenzyme pattern and differentiation of cells was studied in detail with blood and leukemia cells (Cohn *et al.*, 1987; Gignac and Drexler, 1990), but few report has appeared on such relation for hepatic cells, except the study on the progress of hepatocarcinogenesis (Maki *et al.*, 1991). A study on tumor-associated protein variations in rat hepatic cells has been recently reported without description about esterase activities (Zeindl-Eberhart *et al.*, 1994). A preliminary electrophoretic analysis of esterases in the rat hepatic and hepatoma-derived cells was reported in the previous paper (Kageyama *et al.*, 1995). More detailed study on this problem by isoelectric focusing (IEF) and two-dimensional polyacrylamide gel electrophoresis (2D PAGE) is described here.

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Materials and Methods

Non-cancerous rat liver cell lines, BRL and ARLJ301–3, and EJ-ras oncogene transformed

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rat liver cell lines, Anr4 and Anr13–1, were received from RIKEN Cell Bank (Tsukuba) and non-cancerous rat liver cell lines, BRL 3A and Clone 9, and rat hepatoma cell lines, H4-II-E, McA-RH7777, and MH_1C_1, from DAINIPPON Pharmaceutical Ltd. (Osaka). The cells were cultured according to the supplier's catalogue. The harvested cells were thoroughly washed with ice-cold phosphate-buffered saline and then stored in a freezer (< -30 °C) until use. Rat liver was taken from a male Wister Rat (4 weeks old).

The pellet of cells (0.04 ~ 0.05 cm^3) of each cell line or a cut of rat liver (0.02 ~ 0.03 cm^3) was disrupted by a Potter-type microhomogenizer with 10 µl digitonin solution (2% in 0.1 M phosphate buffer, pH 7.5). After centrifugation of the suspensions at 15000 rpm for 3 min, the supernatants were used as enzyme extracts.

Density gradient (10/20) precast gels for one- and two-dimensional PAGE were purchased from Daiichi Kagaku Kogyo Ltd. (Tokyo). The enzyme extracts of rat liver, McA-RH7777 cells, and MH_1C_1 cells were diluted 24-, 24-, and 2-fold, respectively, with 0.1 M phosphate buffer (pH 7.5). The enzyme extracts of other cells were used as they were. These samples (1 ~ 3 µl each) were mixed with a glycerol/BPB/Tris·HCl solution (7 µl) and then applied to the wells of gels. The electrophoresis was done with Tris (0.3%)/glycine (1.44%) as the electrode solution at a constant current of 20 mA (per 10 x 10 cm gel) for 4.5 hr. The gels were stained with a mixture of 0.01% (w/v) α-naphthyl acetate and 0.03% Fast Blue RR salt (Sigma Chemical Co., St Louis) in 0.1 M phosphate buffer (pH 6.25) at room temperature normally for 10 ~ 60 min. Lanes with bovine serum albumin (BSA, 20 µg/lane) as M_r markers were stained with Coomassie Brilliant Blue G-250.

Gels for IEF were prepared on supporting films with a casting stand (Model 111 Mini IEF Cell, Bio Rad Laboratories, Hercules, CA) with 2% Bio-Lyte (3/9) according to the Bio Rad's manual. The enzyme extracts were applied to the gel as soaked in filter chips (2 x 5 mm). After 10-min contact, the chips were removed from the surface of gel and the electrophoresis was carried out by impressing the voltage of 100 V for 15 min, 200 V for 15 min, and finally 450 V for 60 min. The gels were actively stained in the same way as described above, or otherwise, the gels as they were adhered to the supporting films were cut longitudinally at the center of each lane to give strips of 5 x 70 mm, which were applied to density gradient (10/20) PAGE for the 2nd dimensional electrophoresis (20 mA, 4.5 hr). Esterases were detected by the activity staining. The pH gradient in the IEF gels was calibrated with an IEF marker protein kit (Oriental Yeast Co., Osaka).

Results

The liver extract gave at least 16 bands in density gradient PAGE, among which 4 bands of 210, 128, 118, and 110 kDa were remarkable (Fig. 1, lanes 2 and 12). Bands of these M_r values were not found in the lanes of the non-cancerous and oncogene-transformed cell lines (lanes 3–8), except that a few lines (*e.g.*, ARLJ301–3, lane 6) weakly exhibited a 118 kDa band. To our surprise, two or three of the above M_r bands were found in the lanes of the hepatoma cell lines (lanes 9–11). Conversely, the major bands in the non-cancerous and oncogene-transformed cell lines (74, 61, and 55 or 47 kDa) were faint in the liver extract and McA-RH7777 and MH_1C_1 hepatoma cell lines (lanes 10 and 11), while they were clear in H4-II-E

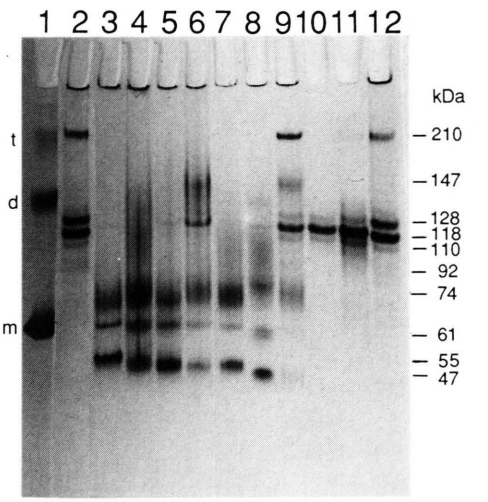

Fig. 1. Esterase zymograms by density gradient PAGE. Samples of lanes: 1, BSA (trimer (201 kDa), dimer (134 kDa), and monomer (67 kDa)); 2 and 12, rat liver extract; 3, BRL 3A; 4, BRL; 5, Clone 9; 6, ARLJ301–3; 7, Anr4; 8, Anr13–1; 9, H4-II-E; 10, McA-RH7777; and 11, MH_1C_1 cell extract. The staining time was 8 min for lanes 2 and 7–12 and 50 min for lanes 3–6.

hepatoma cell line (lane 9). The above remarkable bands in the liver extract also appeared when α-naphthyl butyrate was used as the active staining substrate, but those in the non-cancerous and oncogene-transformed cells became very weak or disappeared with this substrate (data not shown). In addition, the 74 and 61 kDa bands were estimated as 80 and 68 kDa, respectively, in that preliminary electrophoresis (Kageyama *et al.*, 1995).

In IEF (Fig. 2), two prominent bands of p*I* 6.4 and 6.6 were commonly found in the lanes of liver extract and McA-RH7777 and MH_1C_1 hepatoma cell lines (indicated by open arrowheads in lanes 5–7), but they were lacking in BRL, Clone 9 (non-cancerous) and Anr4 (oncogene-transformed) cell lines (lanes 1–3). The p*I* 6.4 band was, though weak, detectable in H4-II-E cell line (indicated by open arrowhead in lane 4). A noteworthy difference between the liver extract and McA-RH7777 cell line is the band of p*I* 6.0 (closed arrowhead in lane 6), which was relatively strong in this cell line but weak in the liver extract. The p*I* values of the normal liver bands were almost in the same range (5.2–6.8) as those reported for several strains of rat (Simon *et al.*, 1985).

The above results indicated that the major bands of the hepatoma cells, McA-RH7777 and MH_1C_1, well corresponded to those of normal liver extract in density gradient PAGE as well as in IEF. To ascertain this, they were compared by 2D PAGE. The major esterase group of M_r ~ 120 kDa in the normal liver extract showed six spots

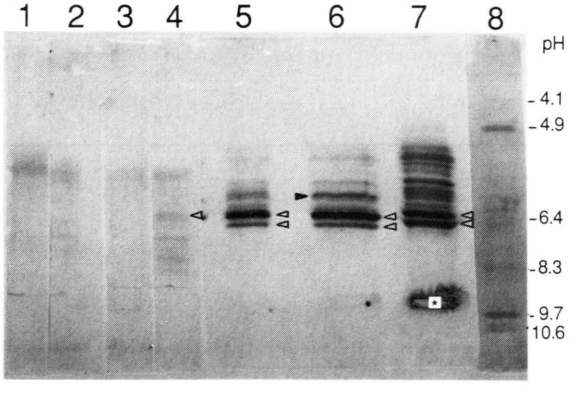

Fig. 2. Esterase zymograms by IEF. Samples of lanes: 1, BRL; 2, Clone 9; 3, Anr4; 4, H4-II-E; 5, MH_1C_1; 6, McA-RH7777; 7, rat liver extract; and 8, p*I* marker proteins. Asterisk in lane 7 indicates the original spotting site in that lane.

in the electrophoretogram, four of which [(p*I* 6.4-M_r 128 kDa), (p*I* 6.6-M_r 128 kDa), (p*I* 6.4-M_r 118 kDa), and (p*I* 6.4-M_r 110 kDa); indicated by arrowheads in Fig. 3a] also appeared in that for McA-RH7777 cell line, but other two spots [(p*I* 6.1-M_r 118 kDa) and (p*I* 6.2-M_r 110 kDa); indicated by arrows in Fig. 3a] were lost and, alternatively, one new prominent spot (p*I* 6.0-M_r 110 kDa) and another new weak spot (p*I* 5.8-M_r 106 kDa) appeared there (Fig. 3b, indicated by arrowheads). Furthermore, the spots of M_r 210 kDa in the liver extract disappeared and the number of spots in the lower M_r region (< 100 kDa) was reduced in this cell line as compared with the liver extract, being consistent with the result in the density gradient PAGE (Fig. 1). Thus, the electrophoretograms in 2D PAGE were considerably different between the normal liver and McA-RH7777 hepatoma cell line.

Discussion

The facts that the established non-cancerous cell lines (BRL, BRL 3A, Clone 9, and ARLJ301–3) gave the esterase zymograms very different from that of the normal liver extract and that the chemically induced hepatoma cell lines (H4-II-E, McA-RH7777, and MH_1C_1) gave rather similar ones seem to imply that the immortalization in *in vitro* culture might cause greater change in the expression of esterase genes than the chemical carcinogenesis. If we cannot accept such an interpretation, we should examine the characteristics of the cell lines. They were all derived from rat liver, but the literature describes BRL and BRL 3A (Perdue *et al.*, 1971), Clone 9 (Weinstein *et al.*, 1975), and ARLJ301–3 (Furukawa *et al*, 1987) cell lines as epithelial cells. Anr4 and Anr13–1 were obtained by transfection of ARLJ301–3 cells with EJ-ras oncogene (Cell Line Catalog of RIKEN Cell Bank). H4-II-E (Pitot *et al.*, 1964) and MH_1C_1 (Richardson *et al.*, 1969) cell lines are also pointed their epithelial morphology, but they were obtained from true hepatomas. McA-RH7777 cell line is described only as rat-hepatoma derived cells, but it produces liver-specific α-fetoprotein and is employed as a hepatocyte model (Wan and Wu, 1992). Perdue *et al.* also described BRL cells as liver parenchyma-like cells, but attempts at hormonal induction of tyrosine aminotransferase (a liver-specific enzyme) were not successful.

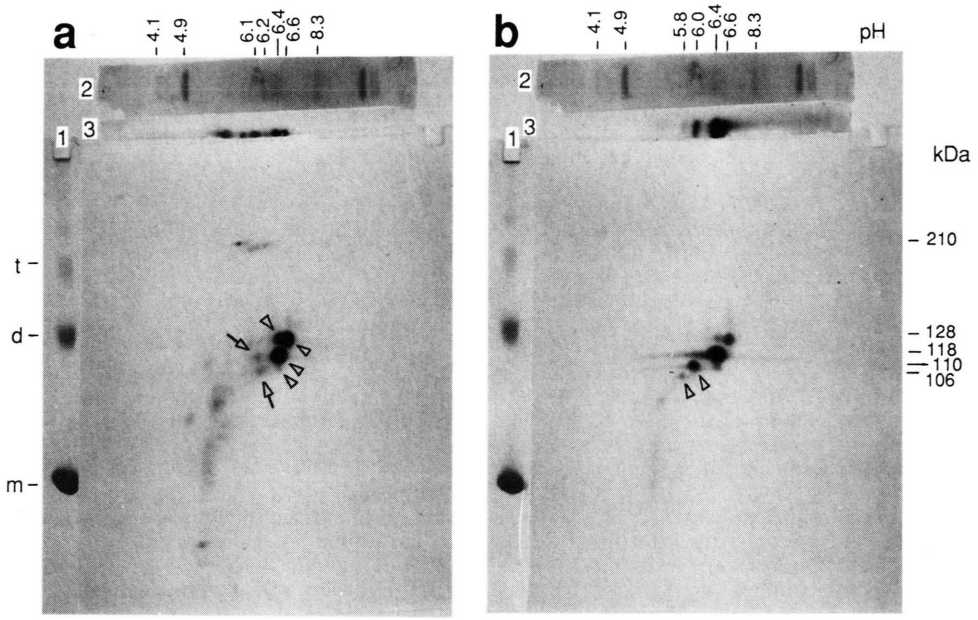

Fig. 3. Esterase zymograms by 2D PAGE of normal rat liver (a) and McA-RH7777 cell extract (b). Lane 1 (vertical) and lane 2 (horizontal) show concomitantly electrophoresed BSA and p*I* marker proteins, respectively. Lane 3 (horizontal) represents a side cut from the original IEF lane. Numbers on the top and on the right side indicate p*I* and M_r values, respectively.

It might be concluded that epithelial cells derived from rat liver other than hepatoma express the 74 ~ 47 kDa esterase bands, which were minor enzymes in the normal liver. On the other hand, the present hepatoma cell lines would keep a part of the characteristics of hepatocytes to express the esterases of M_r ~ 120 kDa very similarly to the normal liver. This should have reflected the contrast between the hydrolytic stereoselectivities of these cell lines (Kageyama *et al.*, 1995).

Furukawa *et al.* suggested concerning the origin of ARLJ301–3 cells that hepatic epithelial cells might have been originated from bile ductular cells. However, the presence of 118 kDa band in the esterase zymogram (Fig. 1, lane 6) implies that the ARLJ301–3 cell line has some characteristic common to the normal hepatocyte, though it was damaged by the oncogene-mediated transformation (Fig. 1, lanes 7 and 8). The zymogram of H4-II-E cell line (Fig. 1, lane 9) suggests that this line seems to express both esterases characteristic of epithelial cells and hepatocytes. Since difference in post-translational modification (*e.g.*, glycosylation) must be also taken into consideration, the variable expression of esterases by different cell lines are complicated phenomena that could not be simply summarized by the presumed origin of cells.

The esterase band patterns of the normal rat liver and the McA-RH7777 hepatoma cells (probably parenchyma-derived hepatoma cells) very closely resembled with each other. This fact illustrates the slight biochemical deviation of cancer cells from normal cells. However, there are also clear differences between them, as shown by the appearance of the new esterase spots in the 2D PAGE (Fig. 3b). An important point is what specificity is held by these enzymes. If their specificities are considerably different from those of other esterases in the normal cells, the information would be useful in preparing ester-type prodrugs targeting the hepatoma cells. Isolation of the enzymes to clarify this point is to be studied in future.

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