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Induction of Glutathione *S*-Transferase P-Form in Primary Cultured Rat Hepatocytes by Epidermal Growth Factor and Insulin

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The effects of epidermal growth factor (EGF) (10 ng/ml) and insulin (100 n*M*) on the expression of glutathione *S*-transferases (GSTs), especially the GST-P form (GST 7-7), were examined in primary cultured rat hepatocytes in serum-free medium. On culture with EGF and insulin, the GST activities towards 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene were transiently decreased on day 2 to 10% of those of freshly isolated hepatocytes and then increased to 60 to 100% of those of freshly isolated cells on day 4. Western blot analysis of GSTs revealed that GST-P, which is not present in freshly isolated hepatocytes, was markedly induced and that GST subunits 3 and 4 of the Mu class also increased after addition of EGF and/or insulin, while the subunits 1 and 2 of the Alpha class disappeared. Northern blot analysis showed that on addition of EGF and insulin the level of GST-P mRNA was also elevated and expressions of the nuclear oncogenes c-*jun* and c-*fos* were enhanced. These results suggest that the enhanced expression of GST-P induced by EGF or insulin in primary cultured rat hepatocytes might be regulated by JUN and FOS proteins.

Key words: Glutathione *S*-transferase — Isoenzyme — Primary cultured rat hepatocyte — Growth factor

Cytosolic glutathione *S*-transferases (GSTs,[4] EC 2.5.1.18) are a family of multifunctional dimeric enzymes. They catalyze the conjugation reaction of reduced glutathione with a number of electrophiles biotransformed from xenobiotics, including chemical carcinogens, and also act as binding proteins for bilirubin, cholic acid, steroid hormones, hematin, and carcinogens.[1)] Rat hepatic GSTs have been studied most extensively, and at least 12 molecular forms (isoenzymes) have been identified and grouped into Alpha, Mu, Pi and other classes or families.[1-3)] There are six major forms of GST in the cytosol of normal rat liver; i.e., GSTs 1-1, 1-2 and 2-2 of the Alpha class and 3-3, 3-4 and 4-4 of the Mu class.[1-3)] We previously demonstrated biochemically and immunohistochemically that the placental form of GST (GST-P), or GST 7-7, which is the only GST of the Pi class in rats, is undetectable in normal rat hepatocytes, but is present at high level in preneoplastic hepatocytes, such as enzyme-altered foci and hyperplastic nodules, and in hepatomas induced by chemical hepatocarcinogens.[2-6)] Furthermore, the results from this and other laboratories have shown that this form is not induced before the appearance of GST-P-positive foci by short-term administration of chemical carcinogens and promoters of hepatocarcinogenesis (see reviews[2, 3)]). The molecular mechanism of GST-P expression during hepatocarcinogenesis has been investigated extensively by Muramatsu and his colleagues.[7-9)] For further studies on this mechanism, a good system of GST-P expression is required. GST-P (7-7) was recently found to be inducible in primary cultured rat hepatocytes,[10-14)] so these cells should be useful for studies on the mechanism(s) of GST-P expression in both normal hepatocytes and (pre)-neoplastic cells. Moreover, although in most previous studies, hepatocytes have been cultured in media containing serum (mainly fetal calf serum), they can be cultured in serum-free medium to facilitate identification of the factor(s) responsible for GST-P expression.

AP-1 (JUN) and FOS, the products of nuclear oncogenes c-*jun* and c-*fos*, respectively, bind to the 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-responsive element (TRE) as *trans*acting factors and activate genes containing this sequence.[15-17)] Muramatsu and his colleagues have identified TRE-like sequences in the enhancer region of the GST-P gene.[7-9)] Accordingly, they supposed that GST-P expression might be controlled by JUN (and FOS) or their gene family products. Consistent with this possibility, they demonstrated that the level of c-*jun* mRNA increases with that of GST-P mRNA in rat livers bearing preneoplastic foci and in hepatomas.[18)] To improve our understanding of the mechanism(s) of GST-P expression, we investigated the effects of growth

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[4] The abbreviations used are: GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; DEX, dexamethasone; EGF, epidermal growth factor; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TRE, TPA-responsive element; TGFβ, transforming growth factor β; EDTA, ethylenediaminetetraacetic acid; GGT, γ-glutamyl transferase; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PKC, protein kinase C.

factors and growth-related factors on the expression of GST-P in primary cultured rat hepatocytes. In this paper, we report that GST-P is significantly induced in serum-free primary cultures of rat hepatocytes by the addition of either epidermal growth factor (EGF) or insulin, and that its induction is associated with enhanced transcriptions of c-*jun* and c-*fos*.

MATERIALS AND METHODS

Chemicals 1-Chloro-2,4-dinitrobenzene (CDNB) and dexamethasone (DEX) were purchased from Wako Pure Chemicals, Osaka. 1,2-Dichloro-4-nitrobenzene (DCNB) was from Nakarai Chemicals, Kyoto. A recombinant human EGF was a gift from Earth Chemical Co., Kobe, and insulin was a product of Sigma Chemical Co., St. Louis, MO. TPA was a gift from Professor Y. Takai, Kobe University School of Medicine. Transforming growth factor β (TGFβ) was prepared from rat platelets as reported previously.[19] Aprotinin, an inhibitor of trypsin-like protease, was obtained from Mochida Pharmaceutical Co., Tokyo. [α-^{32}P]dCTP (3,000 Ci/mmol) was from New England Nuclear, Boston, MA. Nitrocellulose filters were from Bio-Rad Laboratories, Richmond, CA. All other reagents were of analytical grade.

Hepatocyte culture Hepatocytes were isolated from adult male Wistar rats weighing 150–200 g by perfusion of the liver *in situ* with collagenase as reported previously.[20] The cells were suspended at a density of 5×10^5 cells/ml in William's medium E supplemented with 10% calf serum, 1 n*M* insulin and 1 n*M* DEX, and cultured at a density of 6.4×10^4 cells/cm^2 (semi-confluent state) in collagen-coated Falcon plastic dishes at 37°C under 5% CO_2 and 30% O_2 in air. After an attachment period of 1 h, the medium was replaced by William's medium E containing 1 n*M* insulin and 1 n*M* DEX. After 24 h cultivation, the medium was changed to William's medium E containing 10 m*M* nicotinamide and 5 units/ml of aprotinin (standard serum-free medium in this paper) with or without 10 ng/ml of EGF and 100 n*M* insulin, as shown in Fig. 1A. In some experiments, either EGF or insulin (at the same concentrations as above) was added and 1 ng/ml of TGFβ or 100 ng/ml of TPA was added with EGF and insulin to the standard serum-free medium after culture for 24 h (Fig. 1B). These media were renewed every two days. Hepatocytes were harvested with a rubber policeman and stocked at −80°C until use.

Enzyme assays The frozen cells were disrupted by sonication in a solution of 150 m*M* Tris-HCl (pH 7.4), 193 m*M* KCl, 6.25 m*M* dithiothreitol and 5 m*M* ethylenediaminetetraacetic acid (EDTA), and centrifuged at 5,000*g* for 10 min. Samples of the resulting supernatants were taken for assay of γ-glutamyl transpeptidase (GGT). The supernatants were then recentrifuged at 105,000*g* for 45 min and the resulting supernatants were used for the assay of GST and for the Western blotting. GGT and GST were assayed by the methods of Tate and Meister[21] and Habig *et al.*,[22] respectively.

Western blot analysis SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12.5% gel by the method of Laemmli[23] and Western blotting was carried out using the anti-GST 1-2, 3-4 or 7-7 antibody by the method of Towbin *et al.*[24] Protein content was determined by the method of Lowry *et al.*[25]

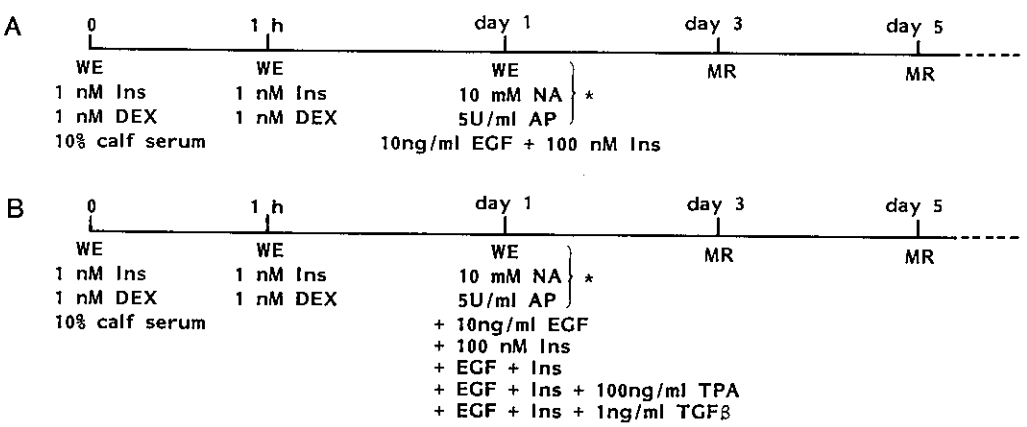

Fig. 1. Summary of primary culture conditions of rat hepatocytes. WE, William's medium E; Ins, insulin; NA, nicotinamide; AP, aprotinin; MR, medium renewal; TGFβ, transforming growth factor β. * standard serum-free medium.

cDNA probes A GST-P cDNA clone (pGP5) was a gift from Dr. M. Muramatsu, University of Tokyo Faculty of Medicine, v-*fos* was supplied by the Japanese Cancer Research Resources Bank, and rat c-*jun* cDNA (pRJ51) was a gift from Dr. M. Sakai, Hokkaido University School of Medicine. These probes were labeled with ^{32}P in a multiprime DNA labeling system (Amersham, UK).

Northern blot analysis Total RNAs were isolated from two or three dishes of cultured hepatocytes by the method of Chirgwin *et al.*[26] Samples of 10 μg of RNAs denatured with formaldehyde and formamide were separated on 1.5% agarose gel containing 0.6 *M* formaldehyde and blotted on nitrocellulose filters.[27] Hybridization was performed in 6×SSC containing 0.1% SDS, 10×Denhardt's solution and 10% dextran sulfate with ^{32}P-labeled cDNA probe at 65°C for 18 h. Then the filters were washed in 1×SSC-0.1% SDS at 65°C, and autoradiographed by exposure to X-ray film (Kodak XAR-5) at −70°C with intensifying screens.

RESULTS

GST activities in primary cultured hepatocytes Rat hepatocytes were cultured at the semi-confluent state in standard serum-free medium with or without EGF and insulin. Then the GST activities of the cells cultured under the two culture conditions towards two substrates, CDNB and DCNB, were compared under both culture conditions (Fig. 2). On day 2 of culture, the activities of cells in both culture conditions towards CDNB and DCNB had decreased to 10% of those of freshly isolated hepatocytes. Thereafter, the activities of cells in cultures in standard serum-free medium alone did not change significantly, but the activities of those in cultures supplemented with EGF and insulin were markedly increased on day 4 to about 60 to 100% of those of freshly isolated hepatocytes, and then decreased slightly. These results indicate that EGF and insulin are effective for the induction of GST activity in primary cultured rat hepatocytes in serum-free medium.

The GST isoenzymes in the cells under these culture conditions were examined by Western blot analysis with anti-GST 1-2 (Alpha class), 3-4 (Mu class) and 7-7 (Pi class) antibodies. As shown in Fig. 3, hepatocytes cultured in standard serum-free medium gave faint bands of GST subunits 1 and 2 of the Alpha class and subunits 3 and 4 of the Mu class and their levels did not change during culture. Subunit 7 appeared at low level on day 6 of culture of cells in standard serum-free medium. In contrast, in medium with EGF and insulin subunits 1 and 2 disappeared and subunits 3 and 4 became expressed more strongly than in medium without EGF and insulin. But the most striking difference was that subunit 7 appeared on day 4 and its level nearly reached that in liver bearing hepatocarcinogen-induced hyperplastic

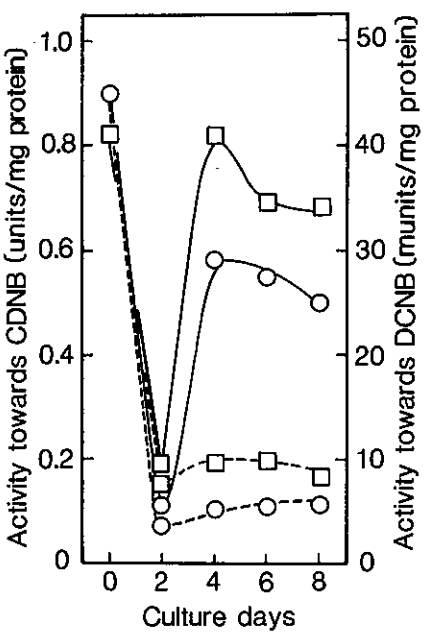

Fig. 2. Changes in GST activities of rat hepatocytes under different culture conditions. Dotted line, culture in standard serum-free medium (William's medium E supplied with nicotinamide and aprotinin); solid line, culture in the same medium supplemented with EGF and insulin. ○ and □, GST activities towards CDNB and DCNB, respectively. For details of culture conditions, see "Materials and Methods" and Fig. 1A.

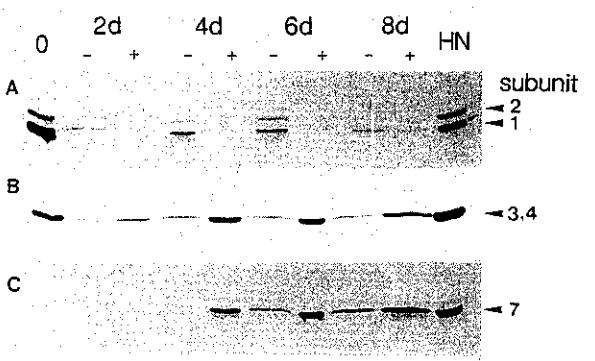

Fig. 3. Western blot analysis of GST subunits separated by SDS-PAGE. Cytosolic protein (50 μg) was applied to each lane. Antibodies to the respective GST forms were used at 2,000-fold dilution. A, B and C show parts of SDS-PAGE gels stained with GST 1-2, 3-4 and P (7-7) antibodies, respectively; 0, freshly isolated hepatocytes; 2d, 4d, 6d and 8d, cells cultured for 2, 4, 6 and 8 days; HN, rat liver bearing hyperplastic nodules; −, cultured in standard serum-free medium; +, cultured in medium supplemented with EGF and insulin.

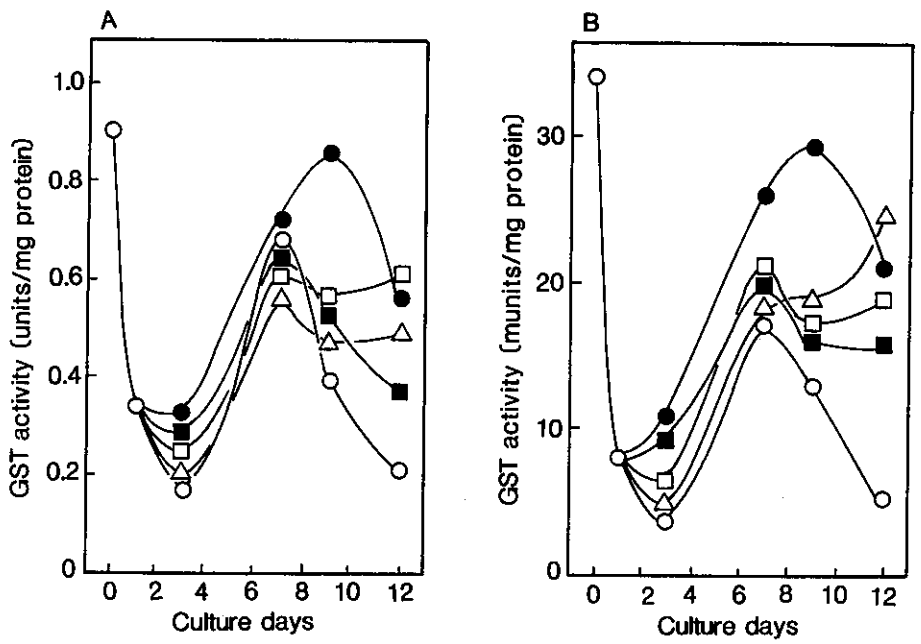

Fig. 4. Effects of growth factors and related compounds on GST activities of primary cultured hepatocytes. A, activity towards CDNB; B, activity towards DCNB. (○), insulin; (□), EGF; (△), EGF+insulin; (●), EGF+insulin+TPA; (■), EGF+insulin+ TGFβ. For additional details, see "Materials and Methods" and Fig. 1B.

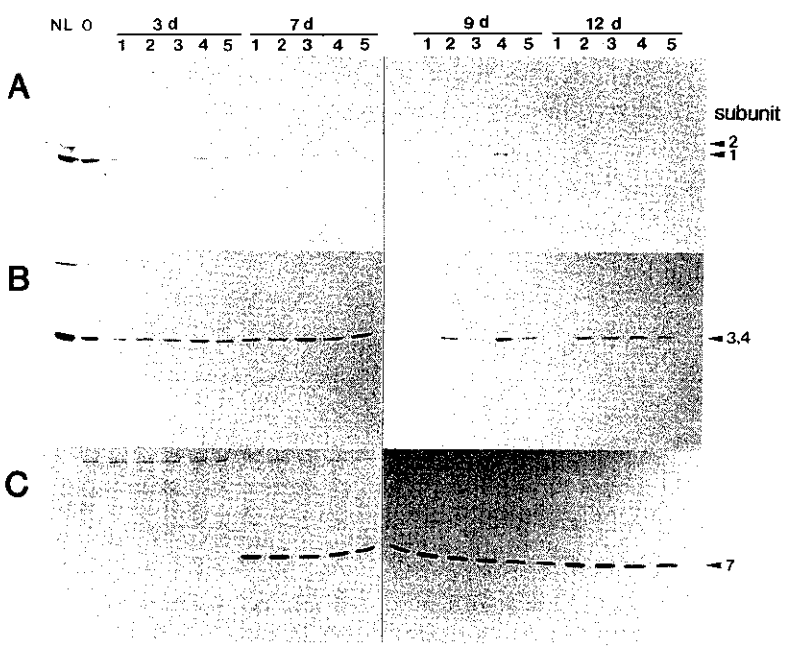

Fig. 5. Western blot analysis of GST subunits induced by growth factors and related compounds. Antibodies to the respective GST forms were used at 2,000-fold dilution. For explanations of A, B and C, see Fig. 3. NL, normal liver; 0, freshly isolated hepatocytes; 3d, 7d, 9d and 12d, hepatocytes cultured for 3, 7, 9 and 12 days; 1, insulin; 2, EGF; 3, EGF+insulin; 4, EGF+insulin+TPA; 5, EGF+insulin+TGFβ.

nodules (Fig. 3). The results in Fig. 3 indicate that the increases in subunits 3, 4 and 7 were responsible for the elevation of total GST activity (Fig. 2) and that EGF and insulin strongly induced GST-P in rat hepatocytes cultured in serum-free medium. The GST-P expression did not seem to be dependent on cell growth, because

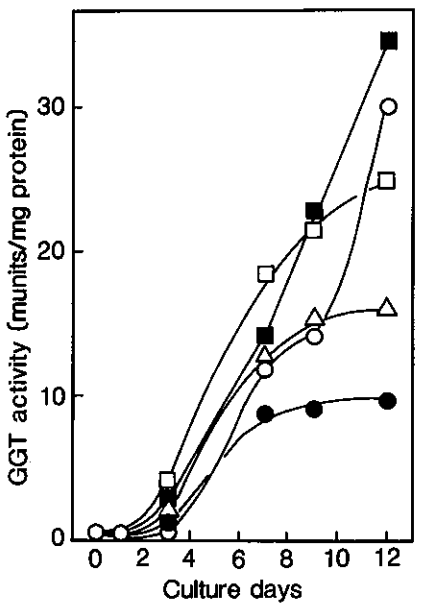

Fig. 6. Effects of growth factors and related compounds on GGT activity. Symbols are the same as in Fig. 4.

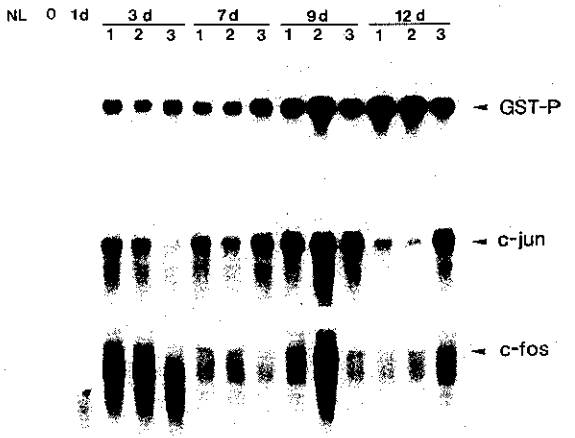

Fig. 7. Northern blot analysis of GST-P (7-7), c-*jun* and c-*fos* in cultured hepatocytes. 1, EGF+insulin; 2, EGF+insulin+TGFβ; 3, EGF+insulin+TPA. Abbreviations used are the same as in Fig. 5.

similar increases in subunit 7 were observed after the same culture periods in semi-confluent cultures and in sparse cultures (2.4×10^4 cells/cm^2), in which the frequency of cell division was high (data not shown).

Effects of EGF, insulin, TPA and TGFβ on GST expression Next we compared the effects of EGF and insulin singly, in combination, and with TGFβ or TPA on the total GST activity and GST-P expression. As shown in Fig. 4, after addition of either EGF or insulin to the medium, the GST activities towards CDNB and DCNB increased to the levels in cultures with EGF plus insulin. However, both activities decreased significantly on long-term culture for 9 to 12 days with insulin alone. Addition of TPA to medium supplemented with EGF and insulin induced further elevation of the GST activities on day 10 of culture. TGFβ, which is known to suppress the growth and functions of various cells, did not significantly inhibit the increase in GST activities towards the two substrates.

As shown in Fig. 5, Western blot analysis revealed that subunits 1 and 2 disappeared during culture under all the conditions examined, while subunits 3 and 4 decreased on long-term culture in medium supplied with insulin alone, suggesting that the decreases in the GST activities in this medium may be due to decreases in subunits 3 and 4. On the other hand, subunit 7 (GST-P subunit) was strongly expressed from day 7 of culture, and its level was almost the same under all the culture conditions examined. These results indicate that GST-P was inducible by either EGF or insulin alone in primary cultured hepatocytes and that this induction was not affected by the presence of TPA or TGFβ.

We found previously that GGT activity increased concomitantly with increase in GST-P during chemical hepatocarcinogenesis in rats.[2,6] To confirm this finding in primary cultured hepatocytes, we also measured GGT. On day 12, GGT activity was highest in cultures with TGFβ together with EGF and insulin, and lowest in those with TPA together with EGF and insulin, as shown in Fig.6. These results indicated that change in GGT activity was not necessarily parallel with those of GST activities towards CDNB and DCNB (Fig. 4) or with the amount of GST-P (Fig. 5) in cultured rat hepatocytes.

Expressions of c-*jun* and c-*fos* The expression of GST-P was compared with those of the nuclear oncogenes c-*jun* and c-*fos* in primary cultured hepatocytes. Northern blot analysis (Fig. 7) showed that c-*jun* and c-*fos* were not expressed in normal liver, freshly isolated hepatocytes from normal liver, or hepatocytes cultures for 1 day, but were both expressed on day 3 of culture with EGF and insulin, when GST-P mRNA also began to be expressed. The expressions of these mRNAs continued until at least day 12 of culture. Fig. 7 also shows that further addition of TPA or TGFβ to medium containing EGF and insulin had no effect on the expressions of GST-P and the oncogenes, These results indicate that the enhancement of GST-P expression by EGF or insulin in cultured hepatocytes was associated with the expressions of JUN and FOS, the products of c-*jun* and c-*fos*. These proteins are proposed to be *trans*acting factors on an enhancer element, i.e. TRE-like sequence in the GST-P gene.[7-9]

DISCUSSION

GST-P (7-7) has been used for analysis of chemical hepatocarcinogenesis in rats and for screening of chemical (hepato)-carcinogens.[2, 3, 28] However, the mechanism(s) of GST-P expression in preneoplastic and neoplastic lesions is not well understood. Recent reports on the expression of GSTs including GST-P (GST 7-7) in primary cultured rat hepatocytes suggest that this *in vitro* system may provide a suitable model for investigating the mechanism(s) of gene expressions of GST-P and other GST forms.[10–14]

In most previous studies, hepatocytes were cultrured in media containing serum. Power *et al.*[10] observed a low level of GST-P expression in an immortalized non-transformed epithelial cell line from rat liver, and a great increase in its level when these cells were transformed by transfection with *ras* oncogenes or treatment with aflatoxin B_1 metabolites. Vandenberghe *et al.*[11] reported reduced levels of GST subunits 1 and 2, and increased levels of subunits 3 and 7 in hepatocytes after culture for 4 days. Abramovitz *et al.*[13] found that Yp (GST-P subunit) mRNA, which is hardly detectable in freshly isolated hepatocytes, rapidly accumulates in the first 24 h during culture of the cells in medium supplemented with bovine insulin, but that its increase is effectively blocked by the presence of DEX. Gebhardt *et al.*[14] also noted that in the presence of DEX, Yp remains at a stable low level.

In this study, we used primary cultures of rat hepatocytes in serum-free culture medium and demonstrated that GST-P expression is enhanced by either EGF or insulin. Therefore, the spontaneous induction of GST-P observed in cultures containing serum may be due to the presence of these hormones in the added serum.

There are reports that EGF and insulin provoke a transient increase of c-*fos* mRNA in rat hepatocytes,[29] rat hepatoma cells and Chinese hamster ovary fibroblasts.[30, 31] There are also reports that EGF stimulates c-*jun* transcription in rat fibroblasts,[32] and that the expressions of the c-*fos* and c-*jun* oncogenes are generally inhibited by a PKC inhibitor.[33] These reports suggest that EGF and insulin may enhance the expressions of c-*fos* and c-*jun* in cultured rat hepatocytes through PKC activation. Rat hepatocytes are known to have high levels of EGF and insulin receptors.[34–36] Sakai *et al.*[7] demonstrated that a TRE-like sequence is aligned in the 5′-flanking region of the GST-P gene. Thus EGF or insulin may promote receptor-mediated PKC activation in cultured rat hepatocytes and cause increased transcriptions of c-*jun* and c-*fos*, and subsequent binding of the JUN/FOS complex to the TRE-like sequence may enhance GST-P expression. Addition of TPA to hepatocytes treated with insulin and EGF caused only a slight increase in GST-P expression, suggesting that the effects of insulin and EGF on PKC resulted in almost maximal stimulation under our conditions.

The expression of c-*jun* in association with that of GST-P was observed at an early stage of chemical hepatocarcinogenesis in rats,[18] and c-*fos* expression was also observed in this stage (our unpublished data). These findings suggest that GST-P expression in preneoplastic hepatocytes might be regulated by JUN and FOS. The present results further suggest that GST-P expression in primary cultured rat hepatocytes might be enhanced by EGF or insulin with co-expression of c-*jun* and c-*fos* without cell transformation. In this connection, previous reports[13, 14] describing the inhibitory effect of DEX on Yp expression in rat hepatocytes are noteworthy, because recently the transcriptional activity of FOS/JUN was reported to be inhibited by the glucocorticoid receptor.[37, 38]

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