EFFECTS OF THYMIDINE AND 2-D-DEOXYGLUCOSE ON INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-1 (IGFBP-1) AND ERYTHROPOIETIN SECRETION IN HEPG2 CELLS

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ABSTRACT

Effects of thymidine and 2-D-deoxyglucose on insulin-like growth factor-binding protein-1 (IGFBP-1) and erythropoietin (Ep) secretion were investigated in HepG2 cells. The maximal insulin-like growth factor-I (IGF-I) binding capacity was obtained at confluency, while Ep production was dominant in the proliferating stage and declined at confluency. Treatment with high concentration of thymidine inhibited Ep production as well as proliferation of HepG2 cells, while IGFBP-1 production was increased by thymidine treatment. Addition of 2-D-deoxyglucose decreased viable cell number and Ep production and increased medium IGFBP-1 levels. These findings suggest that IGFBP-1 and Ep productions are linked to cell proliferation and that IGFBP-1 production is dominant in quiescent cells in HepG2 cells.

Insulin-like growth factor-I (IGF-I) plays a crucial role for fetal development. The biological actions of IGF-I is regulated mainly by binding to IGF binding proteins because growth hormone has a limited role in fetal growth and IGF-I production (4). It has been reported that IGFBP-1 concentration is increased in intrauterine growth retardation (7, 13). The increase in IGFBP-1 could decrease availability of IGF-I and consequently affect fetal development. The mechanisms of the increase in IGFBP-1 levels remain to be elucidated.

HepG2 cell has been a reliable model for numerous hepatocyte metabolic functions, including hepatic protein production and insulin regulation of IGFBP-1 production (5). The cells also secrete erythropoietin (Ep) in an oxygen-dependent manner, indicating the cells possess the nature of fetal hepatocytes.

Here we report the effects of blocking DNA

synthesis with high concentration of thymidine and a consequent inhibition of cell proliferation on the productions of IGFBP-1 and Ep in HepG2 cells as a model of fetal hepatocyte. Effects of low glucose concentration and of 2-D-deoxyglucose on the secretion of these proteins as well as cell proliferation were also studied.

HepG2 cells were obtained from RIKEN cell bank, Wako, Saitama, Japan. The cells were cultured in DME supplemented with 10% FCS, penicillin (100 IU/mL) and streptomycin (100 μ g/mL). In Experiment I, 0.25, 0.5, 1, and 2×10^5 cells/mL were plated on 24-well dishes (1 mL/2 cm²). The culture medium was changed every 2 days. In Experiment II, 0.5×10^5 cells/2 cm² were plated on 24-well dishes. Two days after plating, when the culture was 60–70% confluency, the cells were treated with 0.1, 1, and 10 mM thymidine. The culture medium with or without thymidine was changed every 2 days. At the designated days, the conditioned medium was aspirated and stored at -20°C for IGFBP-1 and Ep assays, and the remaining cells were submitted to determination of viable cell numbers. In Exper-

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iment III, two days after plating at the density of 0.5×10^5 cells/ 2 cm^2 , FCS was substituted with 0.1% BSA, and the cells were further cultured for 48 hours. The serum-free medium contained 25 mM glucose, 5.5 mM glucose, or 5.5 mM glucose plus 5.5 mM 2-D-deoxyglucose. The medium was aspirated and stored for IGFBP-1 and Ep measurements. Viable cell determination was undertaken in serum-free medium containing 25 mM glucose.

To estimate viable cell numbers, 0.125 mL 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL) was added to the culture (0.5 mL/ 2 cm^2) and was incubated for 4 h. Then, the cells were lysed by addition of 0.5 mL lysis buffer (50% *N*, *N*-dimethylformamide, 20% SDS, 2% acetic acid, 0.025 N HCl, pH 4.7) and incubation at 37°C for 16 h. The mixture (0.225 mL) was transferred into 96 well plates and the absorption at 550 nm (background: 620 nm) was obtained by a spectrophotometer.

HepG2 cell-conditioned medium (0.1 mL) and ^{125}I -IGF-I (10,000 cpm) were mixed in 50 mM Tris-HCl containing 0.5% BSA (pH 7.4) in a final volume of 0.5 mL, and were incubated at 4°C for 16 h. One mL of 1% activated charcoal was added and the mixture was further incubated at 4°C for 30 min, followed by centrifugation at 3000 rpm at 4°C for 30 min. The radioactivity of the supernatant was counted by a gamma counter to determine bound ^{125}I -IGF-I. Non-conditioned medium was assayed in parallel and was subtracted from the bound radioactivity to determine specific IGF-I-binding activity.

IGFBP-1 and Ep levels were determined by a commercial IRMA kit (Diagnostic Systems, Webster, Texas, USA), and a RIA kit (Nippon DPC Co., Tokyo, Japan), respectively, according to the manufacturers' recommendations. Standard preparations were dissolved with non-conditioned medium to exclude non-specific bindings. Thymidine and glucose concentrations in the sample did not affect the assays.

Statistical significance was evaluated by analysis of variance in combination with Duncan's new multiple range test. A *P* value less than 0.05 was considered significant.

As shown in Fig. 1, viable cell number and medium IGF-I binding capacity were dependent on cell density, and the maximal IGF-I binding capacity was obtained at confluency. In contrast, Ep production was dominant in the proliferating stage and declined at confluency. Treatment with

high concentration of thymidine inhibited Ep production as well as proliferation of HepG2 cells in a dose-related manner, while IGFBP-1 production was increased by thymidine treatment (Fig. 2). As shown in Fig. 3, cell proliferation and Ep production were decreased at the glucose concentration of 5.5 mM than at 25 mM. Medium IGFBP-1 level was 80.9% of those at 25 mM, while viable cell numbers were 56.6%. Addition of 2-D-deoxyglucose (5.5 mM) further decreased viable cell number and Ep production (Fig. 3). In contrast, medium IGFBP-1 level was rather increased by 2-D-deoxyglucose (Fig. 3).

HepG2 cells have been proved to be a useful model for studies of Ep and IGFBP-1 biosynthesis. Previous findings that Ep production was oxygen-dependent and that IGFBP-1 production was under a negative control by insulin (5) suggest that physiological mechanisms regulating Ep and IGFBP-1 biosynthesis are operating in the cells. In this study, we found that Ep production by HepG2 cells was prominent at the proliferating phase and declined after the culture reached confluency. Hagiwara *et al.* (8) observed that Ep production by human renal carcinoma cells showed a marked increase after the culture reached confluency. Thus regulating mechanisms of proliferation- or differentiation-associated Ep production are different among cell types. In contrast to Ep, IGFBP-1 production of HepG2 cells was maintained after confluency. These observations

Fig. 1 Effect of cell density on viable cell number (a), medium IGF-I binding capacity (b), and Ep (c) levels in HepG2 cells. The cells were plated at the densities of 0.25 (open square), 0.5 (closed triangle), 1 (open circle), or 2 (closed circle) $\times 10^5/2 \text{ cm}^2$ on day 0, and the culture medium was changed every 2 days. The IGF-I binding activity and Ep concentration in the medium were determined as described in Methods. The cells were submitted to MTT assay. Mean $\pm$ SEM values of 4 samples are shown.

Fig. 2 Effect of thymidine on viable cell number (a), immunoreactive IGFBP-1 (b), and Ep levels (c) in HepG2 cells. The cells were treated with 0.1 (closed diamond), 1 (open circle) or 10 mM thymidine (closed triangle) on day 0. Open square indicates a control. Mean values of 4 samples are shown. *: $P < 0.01$.

Fig. 3 Effects of glucose concentrations and 2-D-deoxyglucose on viable cell number (a), medium IGFBP-1 (b), and Ep levels (c) in HepG2 cells. The cells were cultured for 48 hours in serum-free medium with 25 mM glucose (open bar), 5.5 mM glucose (hatched bar), or 5.5 mM glucose plus 5.5 mM 2-D-deoxyglucose (closed bar). Mean $\pm$ SEM values of 4 samples were shown. *: $P < 0.05$, **: $P < 0.01$.

suggest that Ep and IGFBP-1 productions are regulated by cell machinery in HepG2 cells and does not solely reflect the cell viability or the activity of non-specific protein synthesis.

Effects of cell proliferation on Ep and IGFBP-1 secretion were studied in HepG2 cells treated with thymidine and 2-D-deoxyglucose in the present study. High concentration of thymidine inhibits DNA synthesis (2) through inhibiting ribonucleotide reductase (3). Both treatments decreased Ep secretion as well as cell proliferation during 2 days. Interestingly, we found that IGFBP-1 production was increased by thymidine treatment. IGFBP-1 production was clearly increased in the

presence of 2-D-deoxyglucose. Lowering glucose concentration (5.5 mM) alone slightly decreased medium IGFBP-1 levels while viable cell numbers were markedly decreased, suggesting an increase in IGFBP-1 secreted per cell. Cotterill *et al.* (6) previously reported that glucose concentrations at 4–12 mM were stimulatory but a lower concentration (1 mM) was rather inhibitory on IGFBP-1 production in HepG2 cells. Because their findings were obtained in the experiments over 6 h period in confluent cells, it is plausible that the data mainly reflected metabolic effects of medium glucose concentrations but not effects related to cell proliferation. Lindgren *et al.* (10) observed that 2-D-deoxyglucose at doses of 1 to 10 mM decreased IGFBP-1 secretion in the absence of glucose. Again the incubation time was 15 h, and the viable cell numbers were not evaluated in their study. The observation of Mohn *et al.* (11) that insulin treatment of H35 rat hepatoma cells induced cell proliferation and rapid decrease in IGFBP-1 expression support our hypothesis that IGFBP-1 biosynthesis is linked to cell proliferation. There might be a complex regulation of IGFBP-1 gene expression because IGFBP-1 gene was one of the immediate-early genes specifically expressed in regenerating rat liver *in vivo* (11). Further studies are necessary to clarify reasons of the discrepancy between the *in vivo* and *in vitro* findings.

Several intracellular factors are known to influence IGFBP-1 production in HepG2 cells. It was demonstrated that protein kinase C played a tonic inhibitory effect (9) and that cycloheximide-sensitive labile proteins might contribute to maintain IGFBP-1 promoter activity and turnover of IGFBP-1 mRNA (12). However, the cellular mechanisms linking cell proliferation and IGFBP-1 production remains to be elucidated. It has been reported that IGFBP-1 levels in human amniotic fluid were negatively correlated with lecithin to sphingomyelin ratios, an index of fetal maturity (1). In intrauterine growth retardation associated with maternal diabetes mellitus, IGFBP-1 concentrations in the cord sera were higher than control (7). It was also reported that serum IGFBP-1 levels were increased in small for gestational age fetal rats (13). Taken together, it might be possible that an increase in IGFBP-1 levels in intrauterine growth retardation result from the increase in hepatic IGFBP-1 biosynthesis associated with growth impairment of the liver.

In conclusion, it was suggested that IGFBP-1 and Ep productions are linked to cell proliferation in HepG2 cells. It might explain the increase in IGFBP-1 in the fetus with intrauterine growth retardation.

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