Intermittent addition of HGF and TGF- β 1 in rat primary hepatocyte culture

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Abstract

The effects of hepatocyte growth factor (HGF) and transforming growth factor-beta (TGF- β) on two morphological states of hepatocytes in monolayer and spheroid cultures, were examined in terms of their mitogenic ability and albumin expression. In monolayer culture on collagen-coated dishes, the increase in DNA content in the presence of HGF was observed when HGF was added within two days of cell isolation, whereas no increase in DNA was observed when HGF was added four days of cell isolation. DNA content increased even after four days, when HGF was added intermittently. On the other hand, spheroid formation was promoted on Primaria[®] dishes in HGF-free culture, whereas it was inhibited following the addition of HGF. No increase in DNA content was observed in spheroid cultures even in the presence of HGF throughout the culture period. The albumin production ability rapidly decreased in monolayer culture, but the decline was attenuated following the addition of HGF during the course of culture. A high albumin production ability was maintained independent of HGF supplementation in spheroid culture. Both DNA content and albumin production decreased rapidly following the addition of TGF- β 1 in monolayer culture, and this decline was also attenuated following the addition of HGF to the medium.

Abbreviations: HGF, hepatocyte growth factor; TGF, transforming growth factor; EGF, epidermal growth factor

Introduction

A bioartificial liver support system, in which living hepatocytes are packed in a bioreactor, has attracted a great deal of attention for the treatment of patients with fulminant liver failure. Because liver sustains various life-supporting functions such as detoxification and specific protein synthesis, and cannot be replaced entirely by artificial devices, living hepatocytes are used for an artificial liver. The culture conditions for hepatocyte culture are among the most important factors determining cell viability and expression of liver-specific functions. In this regard, many researchers have investigated culture conditions for hepatocyte culture (Enat et al., 1984; Dich et al., 1988; Ben-ze'ev et al., 1988; Hansen et al., 1994). The results revealed that not only components of the culture medium, such

as growth factors and hormones, but also cell morphology depending on the culture substratum significantly influence the expression of liver-specific functions. In particular, the culture method for three-dimensional culture with dense aggregation of hepatocytes to form spheroids offers promise for the construction of a bioartificial liver, since liver-specific functions are highly expressed even under *in vitro* culture condition (Koide et al., 1990; Matsushita et al., 1991; Tobe et al., 1992; Sakai et al., 1992; Wu et al., 1996; Yamada et al., 1998).

It is well-known that hepatocytes grow actively *in vivo* during the process of regeneration of liver and the growth ceases after the original size of the liver is regained. However, *in vitro* culture of hepatocytes is difficult and the cells do not grow under normal culture conditions. Epidermal growth factor (EGF),

alpha-fibroblastic growth factor (α -FGF), hepatocyte growth factor/scatter factor (HGF/SF) and transforming growth factor-alpha (TGF- α) have been reported as growth factors for stimulating hepatocyte growth *in vitro* (Fausto, 1991). The effects of these mitogens are mediated with hormones such as insulin and glucagon (Fausto, 1991). Among the growth factors, HGF which is secreted during liver regeneration *in vivo* and promotes the growth of hepatocytes, has attracted attention in the development of liver and other organs in recent years (Nakamura et al., 1989; Rubin et al., 1993; Santos et al., 1994; Schmidt et al., 1995; Uehara et al., 1995). When the liver is damaged, HGF is produced from non-parenchymal cells in the liver and other organs such as lung, kidney and spleen, followed by the induction of hepatocyte growth. After sufficient growth is achieved, transforming growth factor-beta (TGF- β) is converted to an active form, and the growth of hepatocytes is terminated (Matsumoto et al., 1992). Cell-cell or cell-organ interactions mediated by the growth factors and hormones are closely correlated to the process of regeneration of the liver *in vivo*.

Thus, HGF and TGF- β are considered to play an important role for the growth and differentiation of hepatocytes. If the growth and differentiation of hepatocytes *in vitro* can be controlled by the use of growth factors and hormones, the construction of a novel bioartificial liver support system may be possible. In the present study, we examined the effects of intermittent addition of HGF and TGF- β 1 on both monolayer and spheroid cultures of hepatocytes.

Materials and methods

Isolation of hepatocyte

Hepatocytes were isolated from 6–7 week-old Sprague-Dawley rats using the *in situ* collagenase-perfusion method described by Seglen (1976) with slight modification. The liver was first perfused via the portal vein with a pre-perfusion solution consisting of phosphate buffered saline (0.151 g/l Na_2HPO_4 , 0.078 g/l NaH_2PO_4 , 8 g/l NaCl and 0.1 g/l KCl, pH 7.4) supplemented with 0.19 g/l EGTA (ethylene glycol bis(β -aminoethyl ether)- N,N,N',N' -tetra-acetic acid), 2.38 g/l HEPES (N -(2-hydroxyethyl) piperazine- N' -(2-ethanesulphonic acid)), 0.35 g/l NaHCO_3 , 0.9 g/l glucose and 0.006 g/l phenol red, for 5 min to wash out blood and to remove calcium ions. It was then perfused with 200 ml of collagenase solution which was similar to the pre-perfusion solution

except that it contained 0.9 g/l collagenase (Wako Pure Chemical Co., Tokyo, Japan) and 0.19 g/l CaCl_2 instead of EGTA and glucose, for 10–15 min to digest liver tissue. The perfused liver was removed from the animal and dispersed by cutting with scissors and gently pipetting in MEM medium (Gibco BRL Co., New York, NY, U.S.A.) supplemented with 5% fetal calf serum (JRH Sciences, Lenexa, KS, U.S.A.). The resultant cell suspension was filtered through a double layer of gauze and a stainless steel sieve (aperture 106 μm) to remove connective tissue. Isolated hepatocytes thus obtained were washed five times with MEM medium by centrifugation at 50 g for 1 min and resuspended in the culture medium. More than 98% of the isolated cells were hepatocytes as determined by phase-contrast microscopy and more than 80–90% were viable as assessed by trypan blue dye exclusion method.

Cell culture and medium

Williams' E medium, supplemented with 0.1 mM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 25 nM Na_2SeO_3 , 1.0 mM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 mM insulin, 1.0 mM dexamethasone, 20 ng/ml EGF, 48 mg/l gentamicin sulfate, and 100 mg/l chloramphenicol was used as culture medium. In some cases, 10 ng/ml HGF (R&D systems Co., Minneapolis, MN, U.S.A.) or 0.5 ng/ml TGF- β 1 (Austral Biologicals Co., San Ramon, CA, U.S.A.) was added to the medium. The cells were seeded at approximately 5×10^5 cells per a 35 mm-diameter plastic dish in 2 ml medium. Collagen-coated plastic dishes (catalog no. 4000-010; Iwaki Glass Works Co., Tokyo, Japan) were used for monolayer culture of hepatocytes and Primaria[®] plastic dishes (catalog no. 3801; Becton Dickinson Co., Bedford, MA, U.S.A.), whose surface is modified with positive charges, were used for spheroid culture of hepatocytes. Five percent fetal calf serum was added to the medium for the collagen-coated dish cultures, and egg yolk lipoprotein was added to the medium (20 mg/ml) instead of fetal calf serum for Primaria[®] -dish cultures. The cells were cultured at 37 °C under 95% air and 5% CO_2 in an incubator (MCO-175; Sanyo Co., Tokyo, Japan). Three dishes were prepared for each of the measurements and the data were averaged.

Measurement of DNA

The amount of DNA in the cultured cells was determined by the DAPI (4',6-diamidino-2-phenylindole di-hydrochloride) method (Hamada and Fujita, 1983).

Briefly, cells in the culture dishes were carefully washed three times with 10 mM Tris-HCl buffer containing 100 mM NaCl and 10 mM EDTA (ethylene diamine-*N,N,N',N'*-tetra-acetic acid). After adding a small amount of the solution, the cells were collected into test tubes by scraping with a rubber cell-scraper, and disrupted by sonication. A small amount (30–60 μ l) of the sample solution was mixed with 3 ml of 100 ng/ml DAPI (Wako Pure Chemical Co.) dissolved in the washing buffer. The complexation of sample DNA with DAPI was fluorometrically determined by excitation at 360 nm and emission at 450 nm using salmon sperm DNA (Sigma Chemical Co., St Louis, MO, U.S.A.) as a standard.

Measurement of albumin concentration

Albumin concentration in the medium was determined using sandwich enzyme-linked immunosorbent assay (ELISA) described elsewhere (Kamihira et al., 1988). Sheep anti-rat albumin (catalog no. 55729; Organon Teknika Co., Durham, NC, U.S.A.) and peroxidase-conjugated anti-rat albumin (catalog no. 55776; Organon Teknika Co.) antibodies were used for the detection of rat albumin, and purified rat albumin (Sigma Chemical Co.) was used as the standard.

Results and discussion

Morphological observation of hepatocytes on the culture substrata

Figure 1 shows the morphology of hepatocytes on day 4. As seen in Figures 1A–C, hepatocytes formed an extended monolayer, when collagen-coated plastic dishes were used as the culture substratum. The cell attachment was slightly different between TGF- β 1-free and TGF- β 1-containing cultures; the cells stretched more on the surface of the dish in the latter case compared to the former (Figure 1C). On a Primaria[®] dish, the cells were initially weakly attached, agglomerated, and formed densely packed, multicellular spheroids within 3–4 days (Figures 1D and F), as described in the previous reports (Koide et al., 1990). However, when HGF was added to the medium at the initiation of culture, spheroids were not formed (Figure 1E). Since HGF is known as a scattering factor for many types of cells, the cells spread over the surface of the dish even on the Primaria[®] dish.

Effects of HGF addition in hepatocyte culture

We then studied the effects of HGF on cell growth and albumin secretion. Figure 2 presents the changes in DNA content and albumin secretion per unit amount of DNA in a collagen-coated dish culture, in which HGF was added to the medium after the cells were cultured in HGF-free medium for 1, 2 or 4 days. The HGF-free or HGF-containing cultures were also used for controls.

In the case of HGF-free culture, no increase in DNA content was observed and the DNA content decreased after 6 days. When HGF was added at the initiation of culture, the DNA content gradually increased until day 4, and thereafter decreased slightly. When HGF was added one or two days after HGF-free culture, increase in DNA content was still observed. However, no increase in DNA content was observed when HGF was added to the medium after 4 days of HGF-free culture. Thus, growth response to HGF addition was gradually lost during culture. In these cases, cell density increased to a maximum of 25% with HGF addition. On the other hand, the albumin production ability decreased in all the cases, as the culture proceeded. The decrease in albumin production ability was slightly attenuated when HGF was added during the course of culture, and not at its initiation. Hence, HGF not only stimulated growth but also maintained the albumin production ability of hepatocytes in culture. This result is consistent with previous report (Takehara et al., 1992). However, since HGF receptors (c-Met) are down-regulated by their binding to ligands (Tajima et al., 1992), the signal from HGF may be transient. Therefore, intermittent addition of HGF might be more effective for maintaining hepatocyte growth and expression of liver functions rather than continuous feeding in monolayer culture.

The results for the Primaria[®]-dish cultures are shown in Figure 3. The hepatocytes in the dishes formed spheroids except when cultured in the presence of HGF, as described above (Figures 1D–F). In the spheroid culture, the increase in DNA content was insignificant following HGF addition, but the albumin production ability was well maintained compared to monolayer culture. Once hepatocytes formed spheroids, the morphology did not change even in the presence of HGF. On the other hand, in cultures in which the cells did not form spheroids, the DNA content increased until day 4, following the addition of HGF at initiation of culture. These results demonstrate that hepatocyte growth is promoted by the addition of

Figure 1. Morphology of hepatocytes at day 4. Hepatocytes were cultured in collagen-coated dishes (A–C) and Primaria® -dishes (D–F). HGF (B, E) or TGF- β 1 (C, F) was added to the medium at the beginning of the culture. Bar: 100 μ m.

HGF in the stretched monolayer, but suppressed in the high-cell density spheroid cultures, although the liver function in the latter was maintained. Thus, the cell morphology significantly influences the response of the cells to growth factor and the expression of liver functions.

In subsequent experiments, we added HGF at the beginning of the culture, and the medium was changed to HGF-free medium during the course of culture. Figure 4 shows the results for monolayer cultures on collagen-coated dishes. In this culture, the cells were cultured in HGF-containing medium for the first 2 days, after which HGF was omitted from the culture medium. After 1 or 2 days of medium change, HGF was again added to the culture medium. In these cases, the DNA content increased until day 2, remained constant for 1 or 2 days, and thereafter increased again with the addition of HGF to the medium. Time course of the DNA content completely corresponded to the medium change; thus, HGF stimulated cell growth. On the other hand, albumin production decreased gradually independent of HGF addition.

The same culture operation was performed for the Primaria® -dish culture (Figure 4). In this culture, the cells were initially attached to the dish surface and did not form spheroids, because HGF was contained in the medium from the beginning of the culture. By changing the medium to HGF-free medium on day 3 and incubating in the same medium for 2 days, the

cells began to agglomerate and formed spheroids. In the case of HGF-free incubation for only one day, the cells did not form spheroids. Once spheroids were formed following the removal of HGF, the albumin production ability was maintained throughout the culture, whereas it decreased drastically after day 6 when spheroids were not formed.

Our results revealed no difference between monolayer and spheroid cultures in the expression of c-Met (Hamamoto et al, 1998). Therefore, the difference in response to the growth factor may be due to the difference in signal transduction induced by the morphological change.

Effects of TGF- β 1 addition in hepatocyte culture

The effects of TGF- β 1 were also examined. Figure 5 shows the results for monolayer cultures on collagen-coated dishes. It is known that TGF- β 1 suppresses hepatocyte growth and induces liver functions *in vivo*. In the culture condition, the DNA content decreased rapidly in the presence of TGF- β 1 compared with TGF- β 1-free cultures. Furthermore, the albumin production ability drastically decreased. Thus, TGF- β 1 suppressed not only hepatocyte viability but also liver functions in the monolayer culture. However, the decrease in viability and albumin production ability in the presence of TGF- β 1 was recovered by changing the medium to the TGF- β 1-free medium (Figure 5).

Figure 2. Effect of HGF addition on DNA content (A) and albumin production ability (B) of hepatocytes in collagen-coated dish culture. HGF-free (W) or HGF-containing (H) Williams' E medium was used. The bar (—) indicates culture period for one day.

Figure 3. Effect of HGF addition on DNA content (A) and albumin production ability (B) of hepatocytes in Primaria[®]-dish culture. HGF-free (W) or HGF-containing (H) Williams' E medium was used. The bar (–) indicates culture period for one day.

Figure 4. Effect of intermittent addition of HGF on DNA content (A) and albumin production ability (B) of hepatocytes in culture. Collagen-coated dishes (circles) and Primaria® dishes (triangles) were used for the culture. HGF-free (W) or HGF-containing (H) Williams' E medium was used. The bar (-) indicates culture period of one day.

Figure 5. Effect of TGF- β 1 addition on DNA content (A) and albumin production ability (B) of hepatocytes in collagen-coated dish culture. TGF- β 1-containing (T) or HGF-containing (H) Williams' E medium was used. The bar (–) indicates culture period for one day.

Figure 6. Effect of TGF- β 1 addition on DNA content (A) and albumin production ability (B) of hepatocytes in Primaria[®]-dish culture. TGF- β 1-containing (T) or HGF-containing (H) Williams' E medium was used. The bar (–) indicates culture period for one day.

The results for Primaria[®]-dish culture are shown in Figure 6. Spheroids were formed when TGF- β 1 was added to the medium and it seemed that the spheroid formation was further promoted by TGF- β 1. Although the DNA content gradually decreased, the albumin production ability was maintained at the same level, which was very different from the observations in the monolayer culture mentioned above. The albumin production gradually decreased when the medium was changed to TGF- β 1-free medium. On the other hand, albumin production was maintained at a constant level when TGF- β 1-containing medium was used throughout the culture. These results reveal that hepatocytes in spheroid cultures are probably near *in vivo* state and the expression of differentiated functions are stabilized, but their response to growth factors is reduced.

The present study suggests that cell morphology and cell-cell interactions strongly influence the response of hepatocytes to growth factors. Since hepatocytes form an organized structure with adjacent hepatocytes and non-parenchymal cells in normal liver, the response to growth factors may also be controlled by direct cell-cell communication.

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