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Combination of epidermal growth factor and insulin is required for multicellular spheroid formation of rat hepatocytes in primary culture.

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Abstract

We showed that the combination of epidermal growth factor (EGF) and insulin is an essential supplement to Williams' #E medium for the formation of floating multicellular spheroids in primary culture of rat hepatocytes. Isolated hepatocytes assembled to form floating multicellular spheroids within 96 h through transient assembly of monolayer islands within the initial 24 h in dishes coated with liver-derived proteoglycans. However, the assembly of multicellular spheroids was severely suppressed in the absence of either EGF or insulin. The reduction of spheroid assembly was correlated with decreased attachment and subsequent decreased formation of monolayer islands within 24 h. The minimum amounts of EGF and insulin required for the formation of floating spheroids were 1 ng/ml and 0.4 microgram/ml, respectively. These results suggest that the enhancement of hepatocyte attachment provided by the combination of EGF and insulin during the early phase of culture is required for the formation of floating spheroids.

KEYWORDS: multicellular spheroid, epidermal growth factor, insulin, rat hepatocyte, primary culture

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Combination of Epidermal Growth Factor and Insulin is Required for Multicellular Spheroid Formation of Rat Hepatocytes in Primary Culture

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We showed that the combination of epidermal growth factor (EGF) and insulin is an essential supplement to Williams' #E medium for the formation of floating multicelluar spheroids in primary culture of rat hepatocytes. Isolated hepatocytes assembled to form floating multicellular spheroids within 96h through transient assembly of monolayer islands within the initial 24h in dishes coated with liver-derived proteoglycans. However, the assembly of multicellular spheroids was severely suppressed in the absence of either EGF or insulin. The reduction of spheroid assembly was correlated with decreased attachment and subsequent decreased formation of monolayer islands within 24h. The minimum amounts of EGF and insulin required for the formation of floating spheroids were 1ng/ml and 0.4μg/ml, respectively. These results suggest that the enhancement of hepatocyte attachment provided by the combination of EGF and insulin during the early phase of culture is required for the formation of floating spheroids.

Key words : **multicellular spheroid, epidermal growth factor, insulin, rat hepatocyte, primary culture**

Hepotocytes are found in the liver as single cell plates along sinusoids and are organized in a tridimensional lobular architecture. However, hepatocytes in primary monolayer culture exhibit a monolayer assembly. These monolayers have been widely used as an experimental model to investigate hepatic metabolism. It is still difficult to maintain tissue specific functions and morphological characteristics of hepatocytes in primary culture despite the addition of EGF and insulin to the culture media enhance the proliferation (1), anabolism and even survival of hepatocytes (2).

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It has been reported by other investigators that tissue specific gene expression is well preserved in various tridimesional assemblies formed in primary culture (3,4). We also reported that hepatocytes assemble to form tridimensional floating multicellular spheroids (5,6), which retained the differentiated morphological features of hepatocytes (6,7) and tissue specific functions (8). In previous reports, both extracelluar matix-derived non-adherent substrate such as proteoglycans (5) and a hormonally defined medium (9) were used for the formation of spheroids.

In this study the minimum supplements in the hormonally defined medium required for spheroid formation were determined.

Materials and Methods

Dishes and culture substrate. Liver-derived proteoglycans were prepared according to the method previously described (5, 8). Polystyrene 35-mm dishes (Falcon 3001) were coated with proteoglycans isolated from rat liver according to the method described elsewhere (5, 8). Briefly, dishes were coated with liver-derived proteoglycans containing 20 μg hexuronic acid, the major glycosaminoglycan moiety of which is dermatan sulfate, air dried, UV irradiated and washed with sterile PBS immediately prior to use.

Cells and culture. Cells were isolated from Sprague Dawley rats (150–200 g) by liver perfusion (10) with 0.05% collagenase as previously described (5). Hepatocytes were collected by repeated centrifugation at 50 × *g* and adjusted to 3×10^5 cells/ml of culture medium, 1.5 ml of which was inoculated per a dish. Of the inoculated cells, 99% were hepatocytes and more than 90% were viable as determined by phase-contrast microscopy and trypan blue dye exclusion test, respectively. Cultures were incubated at 37°C in a CO_2 incubator. Half the medium was replaced at 6 h after the initiation of culture and at 24 h-intervals thereafter unless otherwise specified. When the cell protein concentration was determined, the conditioned medium was fully replaced 24 h after the dish was washed once with fresh medium.

Medium. A hormonally defined medium that was originally developed by Enat *et al.* (9) and modified by us (8) was used as a standard medium. The standard medium (HDM) consisted of Williams' #E medium containing 100 μg/ml penicillin and 100 U/ml streptomycin as a basal medium, supplemented with 50 μg/ml mouse epidermal growth factor (EGF), 10 μg/ml insulin (Ins), 10 μU/ml human growth hormone (GH), 20 mU/ml human prolaction (Pro), the trace elements (TE), 0.1 μM copper, 5 nM selenium and 50 pM zinc as well as 5 μg/ml linoleic acid. Modified HDMs were also used, in which any of EGF (HDM-EGF), insulin (HDM-Ins), GH (HDM-GH), Pro (HDM-Pro) and all the trace elements (HDM-TE) was absent from the standard HDM or the concentrations of EGF and Ins were changed to 0, 1, 5, and 10 μg/ml (EGF) and to 0.4, and 10 μg/ml (insulin).

Morphology of cells. Cell morphology in the culture was monitored by phase-contrast microscopy, and photographs were taken 24 and 96 h after the initiation of culture. The diameters of 100 spheroids per dish were determined by morphometry under phase-contrast microscope. For light microscopic observation, spheroids formed in 96 h were collected by centrifugation at 1000 × *g* for 5 min, fixed in methanol, embedded in 1% agarose, and reembedded in paraffin. Sections of the specimen were stained with hematoxylin and eosin and observed with a light microscope.

Determination of cell protein. After non-attached cells at 24 h were removed by aspiration and a following wash with fresh medium, the remaining cells were retained for culture. Spheroids formed in 10 ml medium were harvested 96 h later by centrifugation and washed once in PBS. Pelleted spheroids were suspended in 1 ml PBS, homogenized with a glass homogenizer, and sonicated for 3 min on ice. Protein concentration of the disrupted cells was determined by the method of Lowry *et al.* (11) using bovine serum albumin as a standard.

Results

Alteration of spheroid formation in modified HDMs. Isolated rat hepatocytes assembled to form floating multicellular spheroids within 96 h in a liver-derived proteoglycan-coated dish containing standard HDM. Spheroid formation was altered in the modified HDMs. In the standard HDM as well as HDM-GH, HDM-Pro, HDM-TE, the isolated hepatocytes attached and spread within the first several hours, and formed monolayer islands thereafter up to 24 h (Fig. 1a–d). The monolayer islands then assembled to form floating multicellular spheroids by shrinking and detaching until 96 h (Fig. 1a–d). However, formation of a monolayer in 24 h and floating multicellular spheroids in 96 h appeared to be markedly suppressed in either HDM-EGF or HDM-Ins (Fig. 1e and f). No typical spheroids were observed in the basal medium or PBS (Fig. 1g and h).

Size and number of spheroids. It appeared by simple morphometry that fewer and smaller spheroids formed in HDM-EGF or HDM-Ins. To confirm this observation, the protein concentration and diameters of spheroids were determined. The average diameters of the spheroids formed in standard HDM, HDM-GH, HDM-

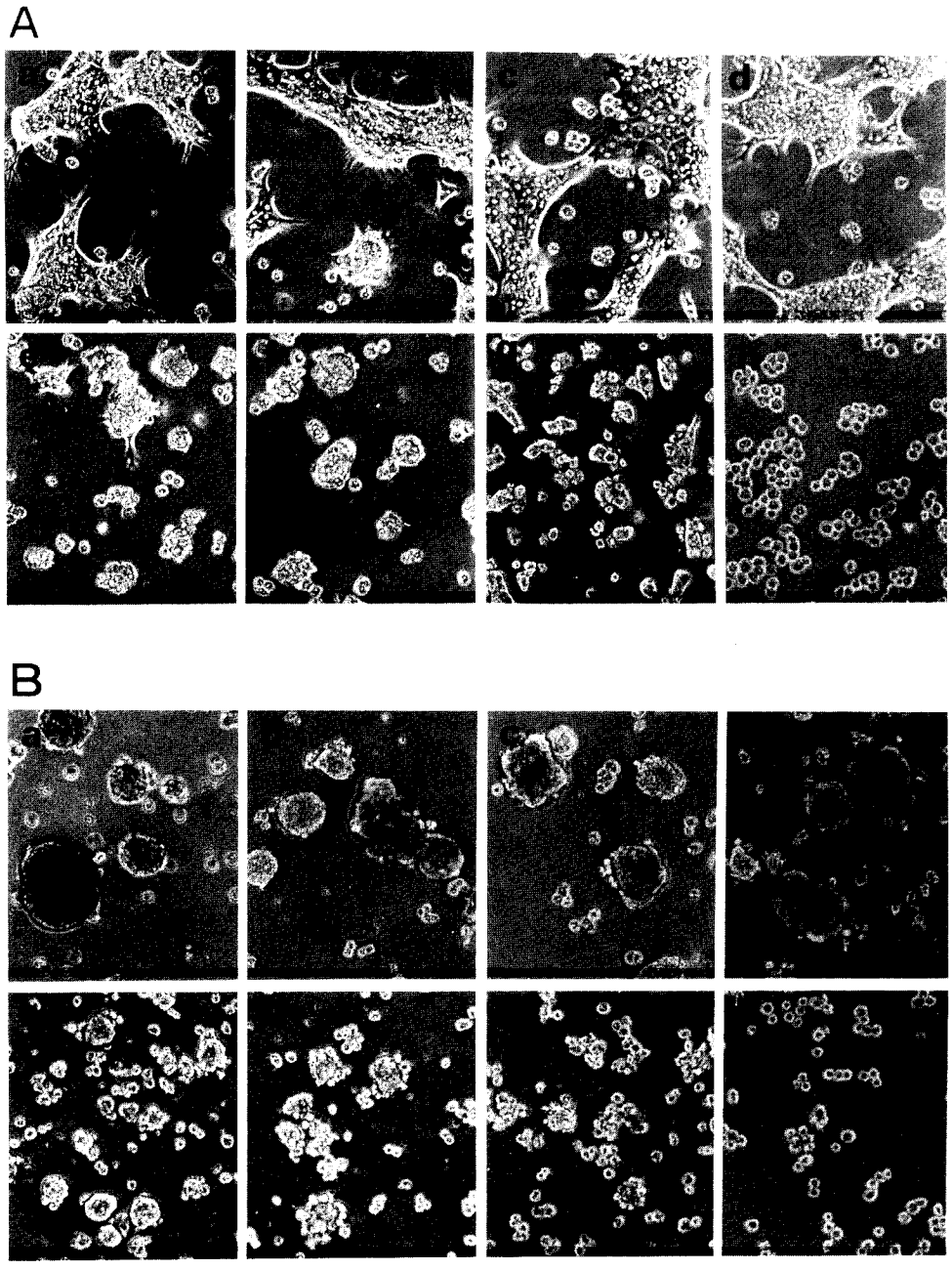

Fig. 1 Assembly of isolated hepatocytes in various hormonally defined media.
The isolated hepatocytes in silgle cell suspension were inoculated into dishes coated with liver-derived proteoglycans. The dishes contained (a) hormonally defined medium (HDM); (b) HDM minus growth hormone; (c) HDM minus prolaction; (d) HDM minus trace elements; (e) HDM minus insulin; (f) HDM minus epidermal growth factor; (g) basal medium; and (h) PBS. Photographs were taken at **A**, 24 h and **B**, 96 h after the initiation of culture.

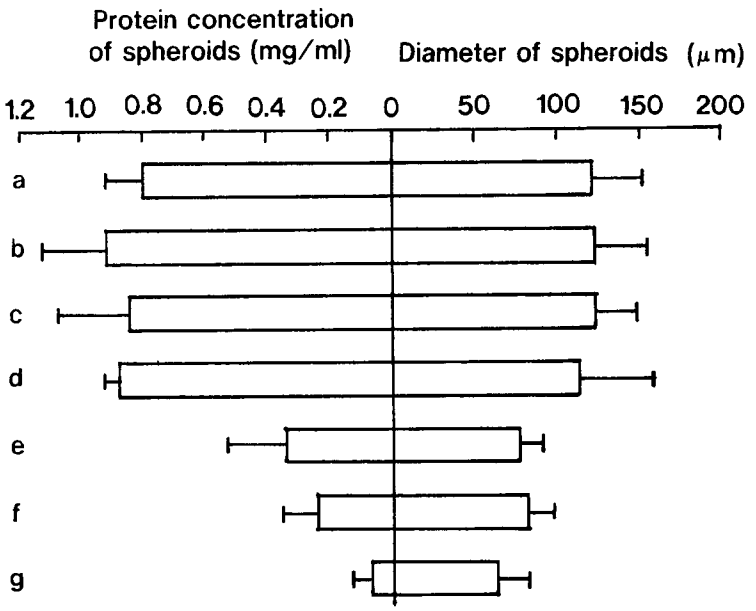

Fig. 2 Diameter and cell protein of multicellular spherods in various hormonally defined media.
Left, Cell protein of multicellular spheroids in HDMs were determined after non-attached cells removed at 24 h. Columns and bars indicate the average values and standard deviations. *Right*, Diameter of multicellular spheroids formed in various HDMs in 96 h was morphometrically determined. Columns and bars indicate the average diameter and standard deviations of 100 spheroids. HDM and marks a, to g correspond to those described in Fig. 1.

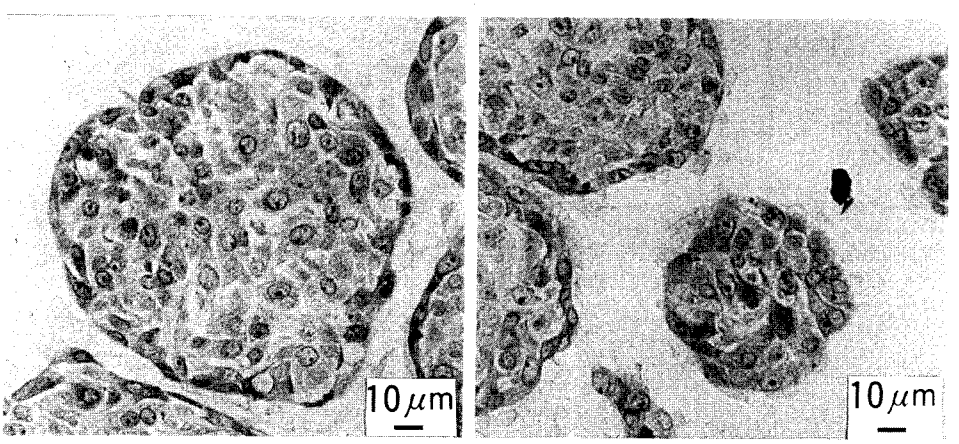

Fig. 3 Microscopic features of sectioned multicellular spheroids.
Multicellular spheroids formed in dishes containing a, HDM and b, HDM minus insulin, were collected at 96 h and processed for the microscopic observation. Bar, 10 μm. HDM: See Fig. 1.

Pro, and HDM-TE were not markedly different, at $122 \pm 25\,\mu$m. Those in HDM-EGF and HDM-Ins, however, were significantly smaller ($p < 0.01$); the average diameters were $80 \pm 11\,\mu$m in HDM-EGF and $76 \pm 14\,\mu$m in HDM-Ins (Fig. 2).

Since most monolayer cells appeared to assemble into floating spheroids, the concentration of protein was determined 96 h after non-assembled single cells at 24 h were extensively removed by washing. The cellular protein in HDM-EGF and HDM-Ins was about 50 % and 25 % of that in the standard HDM, respectively (Fig. 2). It also appeared that the number of spheroids was markedly reduced when the standard HDM was replaced with basal medium.

Morpholgy of multicellular spheroid in HDMs. The spheroids in all of the modified HDMs were viable regardless of their size and amount as determined by trypan blue exclusion, but none of unassembled single cells were. Light microscopy revealed that spheroid cells were viable even in the inner part of spheroids. The only difference between large and small spheroids was the number of cell layers in the multicelluar spheroids; larger spheroids had 5–6 cell-layers, whereas smaller spheroids had 4–5 cell layers (Fig. 3).

Minimum dose of EGF and insulin required for spheroid formation. To determine the minimum doses required for the effective spheroid formation, basal supplemented with either EGF or Insulin were tested. Spheroids were not effectively generated with EGF or insulin alone even at twice the concentrations in HDM. However, spheroids were effectively generated as determined by protein concentration of spheroids when a combination of more than 1 ng/ml EGF and 0.4 μg/ml insulin was supplemented (Fig. 4). The average diameter and protein concentration of the spheroids were almost equivalent to those generated in standard HDM. Therefore, we concluded

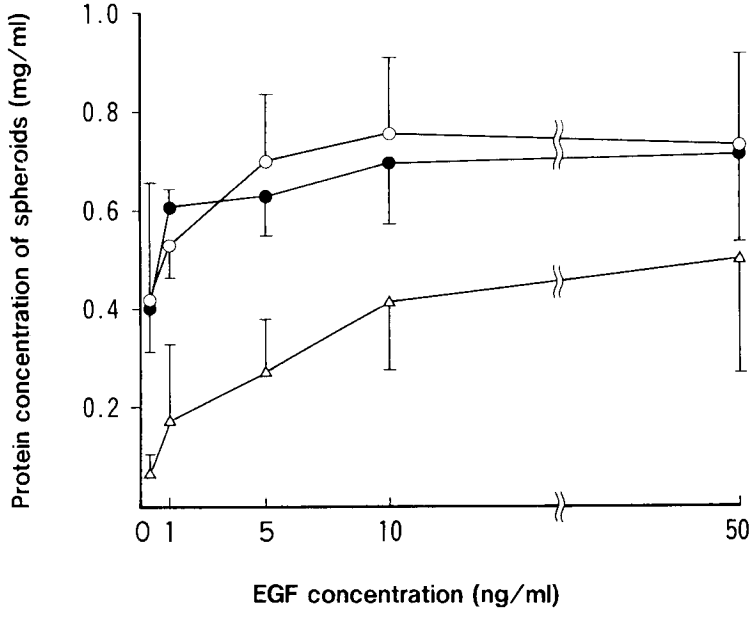

Fig. 4 Effects of various concentrations of epidermal growth factor (EGF) and insulin on protein amount of multicellular spheroids. Multicellular spheroids were formed in dishes containing basal medium supplemented with EGF and insulin. Cell protein of multicellular spheroids formed at 96 h was indicated. Concentration of insulin was △, 0 μg/ml; ●, 0.4 μg/ml and ○, 10 μg/ml.

that the formation of spheroids required the presence of both EGF and insulin in the Williams' #E medium, both of which probably promoted cell attachment under non-adherent environments.

Discussion

We previously described that the formation of spheroids required both non-adherent environments such as those generated by liver-derived proteoglycans in the solid state (5,8) and HDM described by Enat *et al.* (9). In the present study EGF and insulin alone among the supplements in the HDM were responsible for the spheroid formation, and the minimum dose required were 1 ng/ml of EGF and 0.4 μg/ml of insulin respectively. However, either EGF or insulin even at higher concentration did not fully replace HDM, resulting in reduction of number and sizes of spheroids formed. Our results indicate that the reduction of the spheroid formation was directly related to the reduction of the formation of monolayer island in 24 h.

Insulin stimulates anabolism *in vivo* as well as *in vitro*, and EGF stimulates the proliferation of hepatocytes *in vitro*. The attachment of hepatocytes is promoted by insulin in primary cultures (12). Whereas EGF stimulates anabolism in addition to inducing hapatocyte proliferation, the combination of EGF and insulin was more effective in inducing proliferation than EGF alone (13).

One study indicated that hepatocyte collagen production was enhanced as a result of the enhanced utilization of proline in the medium (14). Furthermore, the attachment of hepatocytes to their own collagen may facilitate growth induction. Although collagen production in our culture system remains to be identified, our data suggesed that an adherent environment that promotes attachment and subsequent monolayer formation is generated during the initial 24 h of the process of spheroid formation.

However, replacing precoated liver-derived proteoglycans by any type of collagens; types I, III and IV, did not promote the formation of spheroids even in the presence of HDM, resulting in only the formation of sheet monolayers (6). The addition of serum at concentration above 0.001 % did not promote the formation of spheroids either, even in the presence of precoated liver-derived proteoglycans (15). Since both fibronectin present in serum and collagens are adherent molecules (16), the adherent environments externally introduced by such molecules suppresses the formation of spheroids. The externally introduced adherent environment may simply reduce the non-adherent environment generated by precoated liver-derived proteoglycans. If the combination of EGF and insluin promote the spheroid formation by generating an adherent environment, the phenomenon must only occur in microenvironments.

Recently we reported that precoated liver-derived proteoglycans can be replaced by positively charged polystyrene (9). In positively charged polystyrene dishes, hepatocytes secreted proteoglycans during the early phase of the culture (unpublished data), which may provide a similar environment to that provided by the precoated liver-derived proteoglycans. In addition to proteoglycans, some factor(s) that actively promote the spheroid formation by enhancing detachment of the monolayer islands was present in the conditioned medium of the early culture phase (15). Although we do not know whether the production of the factor(s) is affected, the combination of EGF and insulin was still essential for the formation of spheroids in positively charged dishes. It must be certainly taken into consideration that the combination of EGF and insulin promote the monolayer formation only by stimulating cell anabolism and that the other supplements in the standard HDM faclitate the maintenace of the spheroids after 96 h of culture.

At any rate, since the spheroid is considered to provide a good model to investigate cell-cell adhesion, cell-matrix interaction and tissue orga-

nization *in vitro*, the formation mechanism must be further clarified at the molecular level.

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