Effects of laminin and collagen type I on the morphology and secretion of proteins in human hepatoblastoma and hepatoma cell lines.

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Abstract

The effects of laminin (LAM) and collagen type I (C-I) on human hepatoblastoma (HuH-6) and hepatoma (HuH-7) cell lines were investigated. C-I was superior to LAM in supporting the attachment of the cells, especially of HuH-6, to plastic surfaces. No effect of LAM and C-I on cellular morphology was recognizable by phase contrast microscopy. By scanning electron microscopy (SEM), much more microvilli were found on the cell surface of HuH-6 on LAM substrate than on C-I substrate. In HuH-7 cells, however, these microvilli were rarely found on either LAM substrate or C-I substrate. The gel profile of the proteins secreted by HuH-6 and HuH-7 cells was not affected by the culture substrate except for the major band, though the amount of alpha-fetoprotein (AFP) secreted was larger when the cells were cultured on LAM substrate than on C-I substrate. These results indicate that the ability of LAM or C-I to enhance attachment is different from that to enhance AFP production or microvilli expression in HuH-6 cells and probably in HuH-7 cells.

KEYWORDS: laminin, collagen type I, hepatoma cells, scanning electron microscopy, gel profile

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Effects of Laminin and Collagen Type I on the Morphology and Secretion of Proteins in Human Hepatoblastoma and Hepatoma Cell Lines

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The effects of laminin (LAM) and collagen type I (C-I) on human hepatoblastoma (HuH-6) and hepatoma (HuH-7) cell lines were investigated. C-I was superior to LAM in supporting the attachment of the cells, especially of HuH-6, to plastic surfaces. No effect of LAM and C-I on cellular morphology was recognizable by phase contrast microscopy. By scanning electron microscopy (SEM), much more microvilli were found on the cell surface of HuH-6 on LAM substrate than on C-I substrate. In HuH-7 cells, however, these microvilli were rarely found on either LAM substrate or C-I substrate. The gel profile of the proteins secreted by HuH-6 and HuH-7 cells was not affected by the culture substrate except for the major band, though the amount of α-fetoprotein (AFP) secreted was larger when the cells were cultured on LAM substrate than on C-I substrate. These results indicate that the ability of LAM or C-I to enhance attachment is different from that to enhance AFP production or microvilli expression in HuH-6 cells and probably in HuH-7 cells.

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Human hepatoma cell lines have extensively been used for the study of hepatic functions (1–8). The characterization of these cell lines show them to retain the expression of many liver specific genes in vitro. Our studies have focused on the potential of the hepatoma cells to function as an in vitro model of differentiation (5, 6, 8). Previously, we examined the effects of various extracellular matrix materials (ECMM) on the cultures of human hepatoblastoma (HuH-6) and hepatoma cells (HuH-7) (5, 8). From these studies, it was found that these cell lines were responsive to ECMM in terms of growth and α-fetoprotein (AFP) and albumin production. In this study, we examined the effects of laminin (LAM) and collagen type I (C-I) on the morphology of HuH-6 and HuH-7 cells and gel profiles of proteins secreted by these cell lines, which were not examined in the previous study.

Materials and Methods

Cell culture. Human hepatoblastoma (HuH-6) and hepatoma (HuH-7) cell lines were used (2–8). Both cell lines retain differentiated functions. The population

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doubling times of HuH-6 and HuH-7 are 83.1 h and 46.3 h, respectively. Cells were grown for at least 5 passages in a serum-free medium, RPMI-1640 supplemented with 3 × 10⁻⁹ M linoleic acid, 3 × 10⁻⁸ M oleic acid and trace elements. Cells were maintained at 37°C in a humidified incubator in an atmosphere of 95% air and 5% CO₂.

**Substrate.** LAM (Collaborative Research, Inc., Lexington, Mass., USA) was dissolved in phosphate buffered saline (PBS). C-I (Nitta Gelatin Co., Osaka, Japan) was diluted with 1 : 1000 acetic acid. Multiwell cluster dishes (Falcon, Oxnard, CA, USA) were coated with each substrate, allowed to be incubated at 37°C for 1h and then air dried. Before use, the coated dishes were washed twice with PBS. Coating with each substrate was done at a concentration of 20 μg/ml.

**Attachment rate.** HuH-6 (10⁴ cells/well) and HuH-7 (2 × 10⁴ cells/well) were inoculated into multiwell cluster dishes. Twenty-four h after inoculation, the dishes were washed twice with PBS and the cells were detached with 0.1% trypsin containing 0.025% EDTA in PBS. The cells were counted using a hemocytometer.

**AFP and albumin determination.** HuH-6 (1.2 × 10⁴ cells/well) and HuH-7 (7.5 × 10⁴ cells/well) were inoculated into multiwell cluster dishes. Four days after inoculation, spent medium was processed for the determination of AFP and albumin and the cell number was determined as described above. The concentrations of AFP and albumin were determined by enzyme-linked immunosorbent assay (ELISA) as described before (9).

**Gel electrophoresis.** Five million cells/dish were inoculated into 100 mm Falcon plastic dishes coated with each substrate as described above. Cultures were fed serum-free experimental medium every 2–3 days. One mM sodium-n-butyrate (Ishizu Pharmaceutical Co., Osaka, Japan) was added to the medium fed to the same cultures. Two, 6, 9 and 13 days after inoculation, spent medium was removed, centrifuged at 3000 × g for 10min at 4°C, concentrated 500-fold in collodion bags (Sartorius GmbH, Gottingen, West Germany) and processed for gel electrophoresis. Sodium dodecyl sulf oxide (SDS)-polyacrylamide slab gel electrophoresis was performed essentially as described by Laemmli (10). Gels consisted of a 3% stacking gel and a 10% running gel. The sample buffer contained 1 ml of 0.5 M Tris-HCl, pH 6.8; 1 ml of 45% glycerol; 1 ml of 10% (wt/vol) SDS; 0.4 ml of mercaptoethanol; 0.2 ml of 0.05% (wt/vol) bromophenol blue and 0.7 ml of water. Molecular weight markers (SDS-PAGE molecular weight standards, high and low range; Bio-Rad Laboratories, Richmond, CA, USA) and test proteins were mixed with the same buffer (1:1) and boiled for 3 min in a water bath. Electrophoresis was performed at 200 V, 15 mA for 6h. Gels were stained for protein using Coomasie brilliant blue R-250 (Sigma Chemical Co., St. Louis, MO, USA).

**Morphology.** The monolayer cells, after fixation with 2% glutaraldehyde, were processed by routine methods for scanning electron microscopy (SEM) (JSM-US, Nihon Denki, Tokyo, Japan).

**Results**

Table 1 shows the attachment rate of HuH-6 and HuH-7 cells onto uncoated plastic and the LAM and C-I substrates. The table also shows the amounts of AFP and albumin secreted by the cells when they were cultured on plastic and the LAM and C-I substrates for 4 days. Both HuH-6 and HuH-7 cells adhered most effectively to the C-I substrate, less so to LAM, and least effectively to plastic. This order of effectiveness was much more obvious in HuH-6 cells than in

| Table 1 | The rate of attachment and the amounts of AFP and albumin secreted by HuH-6 and HuH-7 cells on plastic, C-I and LAM
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<tr>
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<tbody>
<tr>
<td>Attachment (%)</td>
<td>AFP (ng/10⁴ cells/4 days)</td>
<td>Albumin (ng/10⁴ cells/4 days)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HuH-6</td>
<td>HuH-7</td>
<td>HuH-6</td>
</tr>
<tr>
<td>Plastic</td>
<td>0.03</td>
<td>30.2</td>
<td>362.4</td>
</tr>
<tr>
<td></td>
<td>(0.02)</td>
<td>(0.6)</td>
<td>(52.6)</td>
</tr>
<tr>
<td>C-I</td>
<td>24*</td>
<td>46.9*</td>
<td>479.7</td>
</tr>
<tr>
<td></td>
<td>(0.2)</td>
<td>(3.0)</td>
<td>(118.2)</td>
</tr>
<tr>
<td>LAM</td>
<td>0.2*</td>
<td>32.4*</td>
<td>657.7*</td>
</tr>
<tr>
<td></td>
<td>(0.04)</td>
<td>(0.9)</td>
<td>(57.8)</td>
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α: Values are average of triplicate samples. Ranges (SD) are given in parentheses. Significantly different from plastic (b, p < 0.001; c, p < 0.05) and C-I (d, p < 0.001; e, p < 0.01; f, p < 0.05), respectively. g, Not done. AFP, α-fetoprotein; C-I, collagen type I; LAM, laminin.
HuH-7 cells. The amount of AFP and albumin secreted in HuH-6 cells was higher, although not always significantly so, when cells were cultured on LAM substrate than on C-I or plastic substrate. In HuH-7 cells, the amount of AFP on LAM substrate was larger than on C-I substrate. However, there was no significant difference between the cells on LAM substrate and those on C-I substrate as to albumin production.

SDS PAGE analysis of serum-free culture supernatant is shown in Fig. 1 (A, B). In our preliminary study, it was found that the position of albumin and AFP was at 66 kDa, suggesting that the major protein band in the gel profile corresponds to albumin and/or AFP. No different gel profiles of the proteins secreted were observed except for the major protein band between the cells on LAM substrate and those on C-I substrate, though new bands appeared as the culture aged and their intensity increased. We have already reported the possible role of sodium N-butyrate and LAM on potentiating differentiation of hepatoma cells (5). In this study, the effect of sodium N-butyrate and substrate on the gel profile of the proteins released was examined. As a result, the intensity of bands was

Fig. 1  Sodium dodecyl sulfate polyacrylamide gel electrophoretic analysis of supernatant from HuH-6 (A) and HuH-7 cells (B).
A. Lane a, low molecular weight standards; lane b, high molecular weight standards; lane c, supernatant on C-I substrate (13 days); lane d, supernatant on C-I substrate (13 days) with 1 mM sodium N-butyrate; lane e, supernatant on LAM substrate (13 days); lane f, supernatant on LAM (13 days) with 1 mM sodium N-butyrate; lane g, supernatant on C-I substrate (2 days); lane h, supernatant on C-I substrate (2 days) with 1 mM sodium N-butyrate; lane i, supernatant on LAM substrate (2 days); lane j, supernatant on LAM substrate (2 days) with sodium N-butyrate. LAM, laminin; C-I, collagen type I.
B. Lane a, low molecular weight standards; lane b, high molecular weight standards; lane c, supernatant on C-I substrate (6 days); lane d, supernatant on C-I substrate (6 days) with 1 mM sodium N-butyrate; lane e, supernatant on LAM substrate (6 days); lane f, supernatant on LAM substrate (6 days) with 1 mM sodium N-butyrate; lane g, supernatant on C-I substrate (9 days); lane h, supernatant on LAM substrate (9 days) with 1 mM sodium N-butyrate. Arrow indicates the position of albumin and α-fetoprotein.
generally reduced by the addition of 1 mM sodium n-butyrate and there was no difference in the gel profiles whether the cells were cultured on C-I substrate or LAM substrate in the presence of sodium n-butyrate.

No distinct morphological differences between HuH-6 and HuH-7 cells on LAM substrate and those on C-I substrate were observed by phase contrast microscopy (Fig. 3).

SEM revealed HuH-6 cells cultured on LAM substrate to exhibit numerous microvilli on the cell surface as compared to those cultured on C-I substrate (Fig. 3). Such an increase in microvilli was not found in HuH-7 cells even when they were cultured on LAM substrate (Fig. 4).

**Fig. 2** Phase contrast micrographs of HuH-6 and HuH-7 cells on various substrates. HuH-6 (A-C) and HuH-7 (D-F) cells were grown on plastic (A, D), C-I (B, E) and LAM (C, F) substrates for 4 days. C-I, collagen type 1; LAM, laminin.
Discussion

Numerous microvilli were observed in the present study when HuH-6 cells were cultured on LAM substrate. Recently, it has been reported that fetal bovine tracheal epithelial cells grown on EHS substrate, which contains predominantly LAM and collagen type IV, have more microvilli than cells cultured on glass or LAM (11). LAM has a more restricted specificity, possibly directed toward epithelial cells, than the other ECMM (8). Thus, it is possible to say that LAM may play an important role in increasing the number of microvilli of epithelial cells. A different response to LAM between HuH-6 and HuH-7 cells was found in this study. This difference may be due to qualitative or quantitative difference in cell surface components that interact with LAM between the two cell lines. LAM mediates epithelial cell binding to collagen type IV (8). Most cells in culture will synthesize ECMM (8, 12). It is also likely that the difference in collagen type IV-producing capacity between HuH-6 and HuH-7 cells is one cause of their different responses to LAM. In this study, the production of AFP was strongly affected by the culture substrate as revealed by ELISA. On the other hand, analysis by SDS PAGE electrophoresis did not show any difference in gel profiles of the proteins, except for the major protein band, which probably represents AFP and/or albumin, between the cells on LAM substrate and those on C-I substrate. Whether LAM or C-I will affect the gel profiles of proteins other than the major protein band in the radiolabelling study remains to be determined. The present result shows that sodium n-butyrate caused a general reduction of the intensity of bands. This could have resulted from decreased protein synthesis, increased protein degradation or interference with the protein secretion process of HuH-6 and HuH-7 cells. The study is in progress to solve this problem, though Nakagawa et al. stated that the last two possibilities appeared unlikely under similar experimental condition using a human hepatoma cell line, PLC/PRF/5 treated with sodium n-butyrate (13).

The present study indicates that ECMM such as LAM or C-I, affects the attachment, the production of AFP and the ultrastructure of HuH-6 or HuH-7 cells and that the ability of LAM or C-I to enhance the attachment is
different from that to enhance AFP production or microvilli expression in HuH-6 cells and probably in HuH-7 cells.

Fig. 4 Scanning electron micrographs of HuH-7 cells on plastic, C-I and LAM substrates. A, Plastic substrate; B, C-I substrate; C, LAM substrate. Bar, 10 μm. C-I, collagen type I; LAM, laminin.

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Effects of Substrates on Hepatoma Cells


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