—Originals—

Laminin-dependent growth arrest of human hepatic carcinoma cell line, HuH-7, in association with expression of p 21/WAF-1 protein

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Abstract

Laminin-overlay to the culture of a human hepatocellular carcinoma cell line, HuH-7, resulted in changes in the cell behavior; suppression of the cell growth, conversion of the cell morphology, and the elevated secretion of cellular AFP in the culture medium, implying that the cells had undergone apparent differentiation in vitro. Together with the behavioral changes, the cells showed positive immunohistochemical staining of the anti-p 21/WAF 1 antibody over the cell nucleus and the amount of p 21/WAF-1 proteins was increased in the cells. p 53 protein was detected both in the control cells and the cells with the laminin overlay. These findings indicate that the laminin-dependent changes in the cell behavior are closely associated with the activation of the cyclin-dependent kinase inhibitor, p 21/WAF-1, and that? is uncoupled with the p 53 expression. (J Nippon Med Sch 1997 ; 64 : 147—153)

Key words: laminin, HuH-7, cells, growth arrest, p 21/WAF-1, p 53

Introduction

Interaction between cells and extracellular matrix (ECM) takes place at the cell surface between the integrin adhesion receptors and the ligand ECM. The adhesion signal leads to changes in the intracellular organization and gene expression of the cells via a seemingly multiple channel of the signal transduction pathway, which involves protein phosphorylation reactions and reorganization of the cytoskeletal elements¹⁻⁶. The response of cells to the signal, regardless of the cells being normal or transformed, makes the cells appear to be in a terminally differentiated state with arrested cell growth, characteristic cell shape, and seemingly ceaseless production of specific proteins⁷⁻¹².

The ECM-dependent changes in the cell behavior, in general, are thought to be reversible processes, and removal of ECM from the cells results in the recovery of cell growth with a loss of the character-

istic cell behavior¹³⁻¹⁵. The ECM-dependent differentiation in vitro thus seems to be somewhat different from the cell differentiation takes place during the developmental process.

Recently, a molecular mechanisms that works in the growth arrest, which is closely associated with terminal differentiation of cells, has been studied by several authors¹⁶⁻¹⁹. Whereas the control of terminal differentiation may be mediated by multiple, possibly redundant, mechanisms, cell cycle arrest through inactivation of cyclin dependent kinase (CdK) is likely to be a central feature. Possible mediators of such negative control are CdK inhibitory (CKI) proteins typified by p 21/WAF-1.

The p 21/WAF-1 protein is thought to function as an inducible growth inhibitor that contributes to cell cycle exit and differentiation. Although transcription of p 21/WAF-1 has been known to be transcriptionally regulated in vitro by the tumor suppressor protein p 53, the expression of p 21/WAF-1 has been found to be independent of p 53 during

terminal differentiation of multiple mouse cell lineages including skeletal muscle, cartilage, skin, and nasal epithelium^{20,21}.

No reports have been published until now concerning the expression of CKI and the tumor suppressor proteins during the growth arrest of tumor cells by ECM. We have described in this paper a study on the expression of p21/WAF-1 and the association of the CKI in laminin-dependent differentiation of tumor cells using a human hepatocellular carcinoma cell line HuH-7.

Materials and Methods

Cell culture and growth: A human hepatocellular carcinoma (HCC) cell line, HuH-7¹⁰, was supplied from Japan Cancer Research Resources Bank. The cells were grown in Dulbecco's modified minimal essential medium/Ham's F12 mixture (DMEM/F12; D-2906, Sigma, St. Louis, MO), supplemented with 10% fetal bovine serum (FBS; GIBCO BRL-Life Technologies, Tokyo) and antibiotics. To assess the growth of HuH-7 cells, the cells in DMEM/F12 with 10% FBS were plated at 1×10^4 cells/well in 4-well multi-dishes (#176740, Nunc, Kamstrup, Denmark) which had been pre-coated with or without laminin. In one experiment, laminin (100 μ g/well) was directly added to the culture medium at 72nd hr of the cell growth. The cells in the wells were harvested with trypsinization and were counted using a Coulter counter. After the laminin supplementation, the cells were counted at 12-hour intervals.

ECM Protein and antibodies: Laminin (mouse laminin; L0001, Iwaki Glass Co., Tokyo) was dissolved in ice-cold phosphate-buffered saline (PBS) before use. Monoclonal antibodies against p21/WAF-1 (#OP 64, Oncogene science, Cambridge, MA), and P53 (#OP 09, Oncogene science) were purchased through the vendors. Anti-mouse immunoglobulin (IgG (γ); #075-1802, Kirkegaard Perry Laboratories Inc, Gaithersburg, MD) was used for a second antibody.

Cell attachment assay: Plates (96 wells; #25801, Corning, Corning, NY) were coated with 100 μ l each of various concentrations of laminin, and incubated overnight at 4°C. The residual binding sites of the wells were blocked with 2% bovine serum albumin

(BSA; BSA-1, United Biotechnological Co., Tokyo). 100 μ l each of the cell suspension, which contained 2×10^4 cells in serum-free DMEM/F12, was seeded in the wells and the plates were incubated in a CO₂ incubator for 2 h. After discarding the spent medium, each well was soaked gently in PBS to remove non-adherent cells. Attached cells were fixed with 10% formalin, stained with 0.2% crystal violet with care not to blot the well-walls, and then washed with tap water. The stained cells were lysed with 2% sodium dodecyl sulfate (SDS) and then absorbency at 595 nm for each lysate was read using a Microplate Reader (Model 450; Bio-rad, Richmond, CA). The adherence activities of the cells were thus expressed in terms of absorbency.

Immunohistochemical staining: HuH-7 cells which had been cultured for 2 days in the medium supplemented with laminin (2 μ g/chamber) in 8-chamber slides (#177402, Nunc) were washed three times with PBS and fixed with acetone. The cells were then stained immuno-histochemically with the monoclonal antibody against p21/WAF1 or p53 using the avidin-biotin-peroxidase complex (ABC) method (Histofine-SAB-PO (M) kit; Nichirei, Tokyo). The cells were counter-stained with Methyl-green.

Western Blotting: HuH-7 cells (1.0×10^6 cells/plate) in culture dishes (#5212, Lux, Miles Scientific, Naperville, IL) were incubated with laminin (100 μ g/ml) for 24 hours. After being washed twice with ice-cold PBS, the cells were lysed in cold lysis buffer consisting of 0.1% sodium azide, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethyl sulfonyl fluoride, 0.15 units/ml aprotinin, 10 mM EDTA, 10 μ g/ml leupeptin, 100 mM sodium fluoride, and 2 mM sodium orthovanadate in Tris-buffered saline (140 mM NaCl, 20 mM Tris-HCl (pH 7.8)) at 4°C for 20 min. The whole cell lysate was centrifuged down at 15,000 rpm for 15 min at 4°C. Protein concentration was determined by Bradford's protein assay method (1). Aliquot of the extract, equivalent to 10 μ g of the protein, was loaded on a gel for Laemmli's SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for 70 min, followed by transfer to a membrane filter (Hybond-C extra; #RPN 203 E, Amersham, Tokyo). The membrane filter was blocked with skim-milk and immersed in the anti-p21/WAF1 antibody solution (1:50 dilution) overnight

at 4°C. The filter was then washed 3 times with 0.2% Tween-20 and incubated with goat anti-mouse IgG (1:2,000 dilution) for 60 min. The antibody-positive band was detected by immuno-reaction using a phosphatase as the substrate.

AFP determination: HuH-7 cells (1×10^4 cells/well) were plated in plastic dishes and were incubated for 48 hr in the medium supplemented with laminin ($10 \mu\text{g}/\text{well}$). The spent medium was collected and the concentration of alpha fetoprotein (AFP) was determined by using an Enzyme Immunoassay (EIA) kit (AFP EIA Onestep-kit, #7224, Dinabot, Tokyo) as indicated in the manufacturer's manual²².

Results

Adhesion activity of HuH-7 cells to laminin was assayed and the result is shown in **Fig. 1**. HuH-7 cells showed positive adherence activity to laminin in a concentration-dependent manner. The cell adhesion was maximum and, in the wells which had been coated with more than $2 \mu\text{g}/\text{well}$ of laminin, it reached a plateau.

Fig. 2a and **2b** show the effects of laminin on the growth of HuH-7 cells. In one experiment, cells were grown on plastic culture plates that had been precoated with laminin (**Fig. 2a**); and in the other

Fig. 1 Attachment assay of HuH-7 to laminin
Points represent the mean $\pm$ SEM.

Fig. 2 Effects of laminin on the growth of HuH-7 cells

a: HuH-7 cells were culture on plastic plates which had been pre-coated with laminin. $\square$; Control cells on the plastic substratum. $\blacklozenge$; Cells on the laminin-coated plates ($10 \mu\text{g}/5 \text{ cm}^2/\text{well}$). b: HuH-7 cells were overlaid with laminin ($10 \mu\text{g}/5 \text{ cm}^2/\text{well}$). $\circ$; Control cells. $\bullet$; Cells with laminin-overlay. The arrow indicates the point at which laminin was added to the culture.

experiment, laminin was directly added to the exponentially growing cells which had been cultured for 3 days on un-coated plastic culture plates (**Fig. 2b**). **Fig. 2a** shows that growth of HuH-7 cells was retarded to a doubling time of 108 h in the early phase of the culture. In the later phase of the culture, the cells retained the normal growth potency with a doubling time of 48 h: identical to the control cells. **Fig. 2b** shows that the cell growth was thoroughly arrested in close association with the addition of laminin the culture medium.

Fig. 3a and **b** show that the addition of laminin to

Fig. 3 Morphological appearance of HuH-7 cells
a: HuH-7 cells on the plastic substratum. b: HuH-7 cells with laminin-overlay. The cell were cultured for 24 hours after addition of laminin ($2\text{ }\mu\text{g}/0.8\text{ cm}^2/\text{chamber}$). The cells show round and flat appearance. (Hematoxylin and eosin stain). $\times 100$.

Table 1 Amounts of alpha fetoprotein (AFP) secreted by HuH-7 cells

AFP(ng/ml/104 cells)	
Control	29.74 ± 4.05
+Laminin	55.35 ± 7.71
Values are mean $\pm$ SD(n=6)	

the exponentially growing HuH-7 cells resulted in marked changes in the morphological appearances of the cells; the cells exhibited round and flat appearances.

To see whether the cells demonstrate characteristic functional differentiation, the spent medium of HuH-7 cells was collected and tested for alpha feto-protein (AFP) secreting activity. In **Table 1** is indicated that the addition of laminin stimulated HuH-7 cells to secrete AFP in the medium. The amount of AFP in the medium of the cells to which laminin had been added was about twice that of the control culture.

The cells cultured in the medium supplemented with laminin were examined by the immunohistochemical ABC-stain method. HuH-7 cells which had been cultured in laminin were stained with anti-p21/WAF-1 antibody reaction products over the

Fig. 4 Immunohistochemical staining of HuH-7 with anti-p21/WAF-1 antibody
a: Control cells cultured on plastic substratum. $\times 100$. b: Cells with laminin-overlay. The cells cultured for 24 hours after addition of laminin ($2\text{ }\mu\text{g}/0.8\text{ cm}^2/\text{chamber}$). The cells are stained positively with the anti-p21/WAF1 antibody. $\times 100$.

Fig. 5 Immunohistochemical staining of HuH-7 cells with anti-p53 antibody
a: Control cells. $\times 100$. b: Cells with laminin-overlay. The cells were cultured for 24 hours after addition of laminin ($2 \mu\text{g}/0.8 \text{ cm}^2/\text{chamber}$). $\times 100$.
The cells are stained with the anti-p53 antibody whether laminin was added to the culture medium or not.

nucleus (**Fig. 4b**); in contrast, the control cells were not stained (**Fig. 4a**), p 53 stains were detected over the nucleus of the cells cultured either in laminin or control medium (**Fig. 5a** and **5b**).

To examine the expression of p 21/WAF 1 proteins, HuH-7 cells were overlayed with laminin for 48 h and then the cell extracts were run on SDS-PAGE, followed by Western blot analysis. In **Fig. 6** is shown that the anti-p 21/WAF 1 antibody-positive band of the protein, of which the molecular weight falls in 21 kD, was apparent in a lane of the extract of the cells with laminin-overlay (Lane 2). The 21 kD-band was seen ambiguously in a lane of control cell extract (Lane 1).

Discussion

HuH-7 cells showed a bi-phasic growth curve

Fig. 6 Western blotting of the HuH-7 cell extracts after SDS-PAGE
Lane 1: Control cell extract. Lane 2: HuH-7 cell extract with laminin-overlay. Anti-p21/WAF1 antibody-positive band is shown. The arrow indicates a band which corresponds to 21 kD protein.

when the cells were plated on laminin-coated culture dishes. Kato, et al²³, reported also that a human colon carcinoma cell line, COLO-201, showed a bi-phasic growth pattern when the cells had been cultured on plates coated with various kinds of ECM. The implication of the results is that at a later stage of culture the cells formed aggregates in which the cells located distantly from ECM received little influence from ECM and regained the anaplastic growth characteristics¹².

Overlaying with ECM resulted in the growth regulation in association with apparent differentiation of the cells in vitro. Tokiwa et al. have reported that enhanced secretion of AFP was detected in the culture medium of HuH-7 cells on laminin-coated plates; however, there were no data concerning the growth of HuH-7 cells on laminin substrate²¹. Roskelley et al. have reported that a clonal population of mouse mammary gland epithelial cells, scp 2, underwent morphological change and expressed milk protein the beta-casein proteins responding to a basement membrane overlay. They defined two components of the cellular response to a basement membrane overlay which caused the cells to express beta-casein gene; one in which changes in organization of the cytoskeletal elements were included and

the other in which protein phosphorylation reactions and beta-1 integrin clustering were involved. Although protein phosphorylation reactions with response to a laminin overlay were shown, no specific regulatory factors that brought about cell differentiation in response to a basement membrane overlay were presented in their report¹⁸. Laminin is one of the main constituents of the basement membrane, and HuH-7 cells responded to a laminin overlay in our present experiments.

The control of cell differentiation has been considered to be mediated by a multiple mechanism, and the factors which cause cell cycle arrest with inactivation of Cdk have been thought to play a central role in the developmental process. Parker, et al. and others have shown that the expression pattern of mouse p 21 correlates with terminal differentiation of multiple cell lineage including skeletal muscle, cartilage, skin, and nasal epithelium in a p 53-independent manner¹⁷.

Expression of p 53 gene regulates the activation of Cdk-inhibitor (CKI) which arrests developing cells progress through the cell cycle and causes cessation of the cell growth. On the other hand, various kind of mutations in p 53 gene have been found in more than half of human cancers, and the mutation in the p 53 gene is thought to be a principal cause of the oncogenic and anaplastic growth of the transformed cells⁹. Hsu. et al²⁵. have already reported that there is a point mutation at codon 220 in p 53 gene of the HuH-7 cell. Furthermore, they have revealed by an immunohistochemical staining method that the HuH-7 cells express the p 53 protein¹⁰.

In our present experiments, however, p 53 expression has been shown in both the control and the experimental HuH-7 cells, and expression of p 21/WAF-1 has been detected in the laminin-overlaid HuH-7 cells. The data imply that a mechanism, in which the activation and/or expression of p 21/WAF-1 is involved and is uncoupled with p 53 expression, works in the process of the laminin-dependent changes in HuH-7 cell behavior.

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(Received, November 18, 1996)

(Accepted for publication, December 16, 1996)