Laminin induces the expression of cytokeratin 19 in hepatocellular carcinoma cells growing in culture

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INTRODUCTION

Cytokeratins (CKs) constitute the cytoskeleton of intermediate filament type in most epithelial cells. They consist of at least 20 members, designated CK1 to CK20 according to their molecular weights and isoelectric points [1,2]. Each type of epithelial cell has a rather stable CK composition, termed CK pattern, which has been used in identification of different epithelial tissues and their neoplasms [3-5]. In normal adult liver, hepatocytes contain only CK18 and CK8 (“hepatocytic” CKs), while the epithelial cells lining the human biliary tree additionally express CK19 and CK7 (“bile duct type” CKs). This difference has been traced back to the early stage of morphogenesis of intrahepatic bile ducts both in rat [6] and in man [7]. It was believed that the characteristic adult CK pattern of each cell type is maintained in various liver diseases including neoplasia (reviewed by Moll et al [8] and Cooper et al [9]). However, CK19 expression, with or without appearance of CK7, has been observed under many pathologic conditions including human hepatocellular carcinoma (HCC) [9,9], chronic hepatitis and cirrhosis caused by alcoholism [10,12], cholestasis [10,12], hepatitis B virus (HBV) infection [13] and exposure of rats to carbon tetrachloride [14,15], butter yellow, and local frostbite injury caused by liquid nitrogen [16] (for more literatures see Van Eyken et al [17] and Fu et al [18]).

The additional expression of CK19 in liver parenchymal cells has been considered a phenotypic change involved in three common pathologic processes, namely 1) remodeling of the liver parenchyma or cirrhotic nodules (destruction of the limiting plate), 2) the capillarization of hepatic sinusoids, and 3) ductular (oval) cell proliferation [19]. However, little is known about the molecular mechanism resulting in CK19 expression under these conditions.

The hepatocytes in normal liver are characterized by the absence of a basement membrane (BM) [5,15,16,19-22] and simplicity of their CK composition [1,17,18]. All the three pathologic processes mentioned above are associated with an increase in production of laminin (LN), a BM glycoprotein, and its deposition within the involved sinusoids to form an LN-positive BM [15,16,23-27]. The abnormal deposition of LN was postulated to be a common cause for CK19 expression in liver parenchymal cells [23,18]. In the present study, we provide direct evidence for this hypothesis in human HCC cell lines using laser-scanning confocal microscopy (LSCM), Western blotting, and an in vitro assay for the induction of CK19.

MATERIALS AND METHODS

Cell lines and cell culture

Six well characterized and intensively used human HCC cell lines, including HepG2 [28], Hep3B [29], HCC-9724 [30,31], HHCC (kindly supplied by Dr. Xian-Hui Wang), HCC-9204 [32,33], and SMMC7721 [34-36], were examined in this study. The cells were cultured in RPMI1640 medium (Gibco BRL, Life Technologies Inc, Gaithersburg, MD, USA) containing 100 mL/L newborn calf serum (Biotech Shaanxi, Xi’an, China), 10² u/L penicillin, 10³ u/L streptomycin and 2 g/L glutamine at 37 °C in the air containing 50 mL% CO₂. After two to three days, the cells monolayers growing on coverslips were taken out from the 12-well plastic flasks, rinsed in 10 mmol/L, pH 7.4 phosphate-buffered saline (PBS) for 2 min and fixed for 10 min in the
methanol/acetone (1:1 in volume) solution precooled at 4 °C. After being washed with PBS for three times, 5 min each, the cell monolayers were ready for the immunostaining as detailed below.

**Immunocytochemical reactions**

The cell monolayers fixed on coverslips were treated in 800 mL/methanol solution containing 3 mL/L H₂O₂ to block the endogenous peroxidase, washed with PBS for three times. After pretreatment with 100 mL/L bovine serum in PBS, separate incubation with monoclonal antibodies to the following proteins was conducted overnight at 4 °C: CK8 (M0631, Dako A/S, Copenhagen, Denmark; 1:40), CK18 (M7010, Dako; 1:40), CK7 (M7018, Dako; 1:40), CK19 (M0772, Dako; 1:75), LN (M0638, Dako; 1:25) and LN receptor (MAB0273, Clone MLUC5, Maxim Bio Fuzhou, China; 1:30). The antigen-antibody conjugation in situ was demonstrated by consecutive incubation with biotinylated anti-mouse/rabbit IgG and streptavidin-labeled peroxidase (S-P) using an Ultrasensitive S-P kit (KIT-9730, Maxim Bio Fuzhou). Finally, the immunoreactions were visualized by incubation in a 3,3’-diaminobenzidine-H₂O₂ solution for 10 min, and the monolayers were slightly counterstained with hematoxylin. Normal mouse and rabbit IgG of the same concentrations were used to substitute for the mouse monoclonal and rabbit polyclonal antibodies, respectively, as negative controls.

**Double immunofluorescence reactions for LN and CK19 and LSCM**

After blocking with 30 mL/L bovine serum in PBS, the cell monolayers were incubated with an antibody mixture (1:1) of mouse monoclonal anti-CK19 (1:75) and rabbit polyclonal anti-LN (Z0097, Dako; 1:38). The reactions were demonstrated by incubation with an antibody mixture (1:1) of the fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Dako; 1:15) and tetramethylrhodamine isothiocyanate (TRITC)-labeled swine anti-rabbit IgG (Dako; 1:30), and observed under a laser-scanning confocal microscope (Zeiss 100) equipped with the Biorad MRC-1024 system. The FITC- and TRITC-labeled signals were visualized at 488 nm and 568 nm, respectively, as their emission wavelength.

**Western blotting analysis**

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blotting were carried out as described previously[19]. Briefly, five cell lines, including HHCC, HCC-9204, HepG2, HCC-9724 and SMMC7721, were cultured in the RPMI1640 medium supplemented with newborn calf serum (100 mL/L). Cells were harvested by digestion with trypsin. For all cell lines examined, 107 cells were used for each protein extraction. The cells were washed in precooled PBS for two times, 5 min each, and then extracted with 1 mL of a Tris-HCl solution (50 mmol/L, pH 8.0) containing 150 mmol/L NaCl, 100 mg/L Nonidet P-40, 50 mg/L Sodium deoxycholate, 10 mg/L SDS, 2 mg/L pepstatin, 2 mg/L Leupeptins and 100 mg/L phenylmethylsulfonyl fluoride. After centrifugation at 2 000 r/min for 2 min, the supernatant was collected. Lysates of the cell lines, 10 µL for each, were loaded on to a 100-g/L SDS-polyacrylamide gel. Proteins were resolved through electrophoresis at 150 V using a miniVE power microscopical fields were determined randomly in ten high-power microscopical fields were determined randomly in different samples, all cells within these areas were counted, and their immunoreactions were evaluated.

In each cell lines immunoreactivities for the antigens detected were graded into negative (-), weakly positive (+), moderately positive (2+) and strongly positive (3+) as described previously[18-20]. For assessment of expression levels of the antigens tested, ten high-power microscopical fields were determined randomly in different samples, all cells within these areas were counted, and their immunoreactions were evaluated.

By incubating the filters in 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) solution for 10 min.

**Induction of CK19 expression by exogenous LN and its blocking test**

Two HCC cell lines, HHCC and HCC-9724, did not produce LN and were negative for CK19 when growing in the ordinary complete medium (see below). They were transferred into the medium containing LN at concentrations ranging from 2.5 mg/L to 60 mg/L. The LN preparation, isolated and purified from mouse Engelbreth-Holm-Swaim tumor, was supplied by Department of Cell Biology, Health Science Center, Peking University. Sterilized PBS of the same volume was used to substitute for LN as a negative control for the assay. After growing for 60 h, the coverslips with cell monolayers within each well were taken out and immunostained for CK19 using the S-P kit as described above.

In order to show specificity of the induction role of exogenous LN, a blocking test was carried out using the cell line, HHCC. The cells were transferred into the complete RPMI1640 medium supplemented with LN (20 mg/L), and then divided into three groups. In one group, the rabbit polyclonal anti-LN IgG, with its concentrations ranging from 20 mg/L to 0.002 mg/L, was added into the LN-containing medium. The two remaining groups, in which normal rabbit IgG at corresponding concentrations or sterilized PBS of the same volume, respectively, were added to the medium, served as negative controls.

**Inhibition test of the CK19 expression associated with intrinsic LN**

In order to elucidate the mechanism of CK19 expression in HCC cells with intrinsic production of LN (see below), SMMC7721 cells were cultured in the ordinary medium without addition of LN, and divided into three groups. As for the blocking test in HHCC, the rabbit anti-LN IgG was added to the culture medium at corresponding concentrations, and normal rabbit IgG or sterilized PBS, respectively, were used for negative controls.

**Statistical analyses**

Intensities of the immunoreaction in single cells were graded into negative (-), weakly positive (+), moderately positive (2+) and strongly positive (3+) as described previously[19-20]. For assessment of expression levels of the antigens tested, ten high-power microscopical fields were determined randomly in different samples, all cells within these areas were counted, and their immunoreactions were evaluated.

In each cell lines immunoreactivities for the antigens detected were graded into negative (-), reaction being absent or too weak to be identified from background, weak (+, definite reaction visible but weak in its intensity or smaller in number of immunoreactive cells) and strong (2+, brown or darker brown reaction in up to half of cells). Statistical computations were conducted using the software package SLPM[41]. The semiquantitative data obtained were analyzed by Karl-Pearson and Krushal-Wallis tests. The P value below 0.05 was regarded as statistical significance.

**RESULTS**

**Expression of CKs, LN and its receptor in different HCC cell lines**

Immunoreactivities for CK18 and CK8 were observed by the immunoperoxidase reaction in cytoplasm of all the six HCC cell lines examined (Table 1). CK7-immunoreactivity was also found in all of these cell lines (Figure 1 A, B), but its intensity varied markedly, being stronger in SMMC7721 and HCC-
9204, and weaker in the others. CK19 and LN expression correlated well, both being present in the cell lines SMMC7721, HepG2 (Figure 1 C, E), Hep3B and HCC-9204, but undetectable in the cell lines HHCC and HCC-9724 (Figure 1 D, F). All of these cell lines were positive for the LN receptor, its immunoreactivity being localized at and beneath the cytoplasmic membrane (Figure 1 G, H).

Expression of CK18 and CK7 was also demonstrated by Western blotting in cell lines SMMC7721, HepG2, HCC-9204, HCC-9724 and HHCC, as shown in Figure 2 A to E, respectively. However, CK19 was only identified in the former three cell lines (Figure 2 A-C), and not in the latter two (Figure 2 D, E).

| Antigens | SMMC7721 | Hep3B | HCC-9204 | HepG2 | HHCC | HCC-9724 |
|----------|-----------|-------|----------|-------|------|----------|
| CK8      | ++        | ++    | ++       | ++    | ++   | ++       |
| CK18     | ++        | ++    | ++       | ++    | ++   | ++       |
| CK7      | ++        | +     | ++        | +     | +    | +        |
| CK19     | +         | +     | +         | -     | -    | -        |
| LN       | +         | +     | +         | -     | -    | -        |
| LN receptor | +    | +     | +         | +     | +    | +        |

Figure 1 Expression of CK7 (A, B), CK19 (C, D), LN (E, F) and its receptor (LN-R, the reactivity denoted by arrows; G, H) in the representative HCC cell lines, HepG2 (A, C, E, G) and HCC-9724 (B, D, F, H). Both CK19 and LN present in HepG2 (C and E) and absent in HCC-9724 (D and F). S-P reaction, slightly counterstained with hematoxylin. ×250.
Figure 2 Western blotting for CK18, CK7 and CK19 using the intermediate filament cytoskeleton extracts from HCC cell lines SMMC7721 (A), HCC-9204 (B), HepG2 (C), HHCC (D) and HCC-9724 (E). Immunoreactions were demonstrated by AP-labeled anti-mouse IgG and visualized in a BCIP/ NBT solution. The right lanes show molecular weight standards (MWS) visualized by staining with Commassie R250, with three indicated by the short bars (from top to bottom, Mr 62 000, Mr 47 500 and Mr 32 500, respectively).

Figure 3 Double immunofluorescence reaction for LN (TRITC-labeled, red) and CK19 (FITC-labeled, green) in HCC cell lines HepG2 (A-C) and HCC-9724 (D-F) under a laser-scanning confocal microscope. Definite signals for LN and CK19, frequently coexisting within cytoplasmic compartment (orange), were found only in the cell line HepG2. ×400.
Concomitant emergence of CK19 and LN as demonstrated by LSCM

CK19 and LN were labeled by double immunofluorescence staining in the same five cell lines showing these proteins on Western blots. The reactions were examined by LSCM, the results being similar to those obtained by the S-P procedure (Table 1). The concomitant occurrence of CK19 and LN was observed in the positive cell lines SMMC7721, HepG2 and HCC-9204, the majority of the positive cells expressing both elements with close correlation of the intensities of the immunoreactivities (Figure 3 A-C). In the cell lines HHCC and HCC-9724, both reactions were negative, showing only some faint background staining (Figure 3 D-F).

Induction of CK19 expression by addition of LN to the medium

The data described above verified the link between the aberrant expression of CK19 in liver parenchymal cells and abnormal LN deposition. However, more direct evidence is needed to unequivocally establish the role of LN for the induction of abnormal CK19 expression. For this reason, an induction test was carried out in two HCC cell lines, HHCC and HCC-9724. Growing of both cell lines in the medium containing LN resulted in the appearance of CK19 when the concentration of LN reached 10 mg/L (Figure 4). The expression level of CK19 was found to increase along with concentration of LN in the medium (Figure 5). In the control group, addition of the same volume of sterilized PBS to the medium did not give rise to any CK19-positive cell.

Blocking of LN-induced CK19 expression by LN antibody

If LN added to culture medium is indeed responsible for the emergence of CK19 expression in the LN-negative cell lines as described above, the effect should be blocked, or at least be partially inhibited, by the addition of LN antibody. This assay was performed using the cell line HHCC. The cells, being negative for both LN and CK19 when growing in ordinary medium, were found to express CK19 with the presence of exogenous LN in the medium (20 mg/L). Its levels in HHCC cells growing in the medium containing different amounts of rabbit anti-LN IgG were assessed. CK19 became undetectable in the cells when the final concentration of LN antibody reached 0.2 mg/L in the LN-containing medium (Figure 6A), while addition of the same amount of normal rabbit IgG or sterilized PBS did not inhibit the expression of CK19 (Figure 6B). These data prove that exogenous LN can induce CK19 expression in HCC cells growing in vitro.
Figure 5  Induction of CK19 expression in HCC cell lines, HHCC (A) and HCC-9724 (B), by LN. Concentrations of LN in culture medium ranging from 2.5 mg/L to 60 mg/L. Numbers of cells expressing CK19 were presented in percentages, and the expression levels indicated by column colors (white, +; gray, 2+; black, 3+). *P < 0.05 (compared to group on the left).

For the HHCC cells whose CK19 expression was completely inhibited by LN antibody, CK19 reappeared when cells were transferred back to the medium containing LN (20 mg/L) and cultured for 14 d. This demonstrates that the inhibition effect by LN antibody is reversible.

The antibody inhibition test was also conducted in the HCC cell line SMMC7721, whose endogenous production of LN has been described above and whose expression of CK19 has been observed when growing in the ordinary medium. Levels of CK19 expression in the cells growing in the medium containing different amounts of rabbit anti-LN IgG were assessed. CK19 became undetectable in the cells when the final concentration of rabbit anti-LN IgG reached 0.02 mg/L in the medium (Figure 7A), while addition of the same amount of normal rabbit IgG or sterilized PBS did not exert this effect (Figure 7B). Similarly, CK19-immunoreactivity reappeared when the cells were transferred to the medium without LN antibody and cultured for 14 d. These data demonstrate that endogenous LN is also responsible for maintaining CK19 expression in HCC cells growing in vitro.

Figure 6  Blocking test of the CK19 expression in HHCC cells induced by exogenous LN. A. CK19 expression completely blocked with concentration of the polyclonal anti-LN up to 0.2 mg/L in the LN-conditioned medium (20 mg/L). B. Addition of the same amount of normal rabbit IgG having no effect on the CK19 expression. Numbers of cells expressing CK19 were presented in percentages, and the expression levels indicated by column colors (white, +; gray, 2+; black, 3+). *P < 0.05 (compared to group on the left).

Figure 7  Blocking test of the CK19 expression in SMMC7721 cells associated with endogenous LN. A. CK19 expression completely blocked with concentration of the polyclonal anti-LN up to 0.02 mg/L in the ordinary medium. B. Addition of the same amount of normal rabbit IgG having no effect on the CK19 expression. Numbers of cells expressing CK19 were presented in percentages, and the expression levels indicated by column colors (white, +; gray, 2+; black, 3+). *P < 0.05 (compared to group on the left).

DISCUSSION

The data obtained in this and other groups during last decade have demonstrated that the CK expression in hepatocytes[10-16,40] and hepatocytic neoplasms[8,9] is not so stable as considered before[17,18]. The expression of CK18 and CK8, the "hepatocytic" CKs, may change greatly in some phenotypically altered hepatocytes[42]. Moreover, expression of CK19, with or without CK7, has been observed in parenchymal cells in some liver diseases, indicating that hepatocytes can also express the so-called "bile duct type" CKs under certain pathological conditions[8,9,17,18]. The abnormal CK19 expression is found in...
chronic hepatitis, cholestasis and cirrhosis[10,15,16], as well as in some HCCs[18,19] and HCC cell lines[9,35,43], and has been considered an adaptive reaction to some alterations of the local microenvironment[15,16,18]. This change has been linked to three common pathologic processes including remodeling of the parenchyma in livers with chronic hepatitis or cirrhosis, capillarization of hepatic sinusoids and ductular (oval) cell proliferation[16]. The ductular (oval) cells are frequently seen in rodent liver under some pathological conditions which suppress regeneration of the parenchymal cells[16,44,45] (for more references see a review by Vessey and de la Hall[50]). Cells with similar morphologic and immunohistochemical phenotypes have also been described in human livers with chronic hepatitis[13,47-50]. In addition, some of the small epithelial liver cells, identified in human livers with chronic hepatitis or cirrhosis and possibly involved in reparative regeneration of liver parenchyma[60] and progression of the preneoplastic foci of altered hepatocytes[42,51], were also shown to express CK19[40,52]. It seems to be true that these CK19-positive cells correspond to a subpopulation of small hepatocytes, being phenotypically intermediate between ductular (oval) cells and typical hepatocytes[48,52]. Considering the frequent abnormal CK19 expression in extrafocal parenchyma in livers with progressive diseases and in HCC cells[16,43,53,54], occurrence of CK19-immunoreactivity in some small-cell preneoplastic foci, as recently observed by Libbrecht et al[59], fails to provide any unequivocal evidence for their speculation that ductular (oval) cells give rise to the small-cell foci, and does not argue against the hepatocytic origin of hepatic preneoplastic foci as demonstrated in both rodents[15,16] and man[42,51,56].

Apparently, the frequent occurrence of CK19 expression reflects the complexity in the differentiation of hepatic epithelial cells, and implies great difficulties in using these “bile duct type” CKs for the differential diagnosis between hepatocellular and cholangiocellular neoplasms[9]. Moreover, its mode of origin has not been fully established. Several lines of indirect evidence have related the aberrant CK19 expression, at least partially, to ductular metaplasia of hepatocytes and glandular differentiation of HCC cells, and indicated its association with abnormal deposition of LN. It has been shown that the morphogenesis of intrahepatic bile ducts in fetal liver, which is characterized by the occurrence of CK19 expression[42], is linked to the deposition of LN around portal vein branches[9]. Secondly, all of the three pathologic processes related to the CK19 expression as discussed above were associated with an abnormal deposition of LN within the hepatic sinusoids involved[15,16,18-27], the patterns of histological distribution of these two immunohistochemical reactions being largely correlated with each other[15,16,27]. Thirdly, both CK19 expression and LN production were also found in some hepatocytes in primary culture and in some HCC cell lines growing in vitro[9,18,35,43,57] and, a coexpression of these two molecules has been noted under these conditions[15,43,58]. The recent data from Blaheta et al[60] and Nishikawa et al[60] proved that fetal hepatocytes growing in culture differentiated in response to some extracellular extracts, containing collagen types I and IV, fibronectin, LN and many other extracellular matrix components. The differentiation was reflected by changes in their CK composition[59-62]. However, these data failed to demonstrate which of the extracellular elements was responsible for the effect.

In this study, the immunocytochemical and Western blotting data proved the concomitant occurrence of LN and CK19 in four of the six HCC cell lines examined, both elements were colocalized in most of the positive cells as demonstrated by LSCM. The data strongly, though still indirectly, suggest a role of LN for the induction of CK19 expression in liver parenchymal cells.

Our induction assay was conducted in two HCC cell lines, HHCC and HCC-9724, which did not produce LN and were negative for CK19 when growing in ordinary medium. CK19 expression emerged with a certain amount of LN added to the culture medium. The effect was found to be dose-dependent, and was completely blocked by a polyclonal antibody against LN. The blocking test was also done with the HCC cell line SMMC7721, which was found to produce LN itself and was positive for CK19 as observed previously[35,43]. LN antibody added to the medium completely inhibited the CK19 expression. These data clearly demonstrate that LN, either of exogenous origin or produced intrinsically, can induce the expression of CK19 in HCC cells in vitro.

Based on results from this and previous studies, we believe that LN, being frequently deposited in hepatic sinusoids as a common response to various kinds of liver injury, is one of the key molecules causing the abnormal CK19 expression. It has been noted that LN is able to induce the hepatocytes to express its receptor, a type of integrin molecules. The receptor takes part in the regulation of cellular differentiation and replication[51-62]. Immuneactivity for LN receptor was also observed in all the six HCC cell lines examined in this study. In addition, the successful blocking of the induction effect of intrinsically produced LN by anti-LN IgG molecules added to the medium, as well as the reversibility of this reaction, indicate that a receptor-mediated pathway is needed for LN to exert its effect. Therefore, we suggest that LN induce CK19 expression in hepatocytes and HCC cells through its receptors located on the surface of their cytoplasmic membrane.

In summary, this study provided direct evidence for an essential role of LN in the induction of CK19 expression in liver parenchymal cells, and established abnormal LN deposition as a key causative factor for the development of aberrant expression of CK19. This will be helpful for the further understanding of differentiation and transformation of liver cells, as well as for evaluating CK19 immunohistochemistry in differential diagnosis of hepatocellular and cholangiocellular carcinomas.

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