Effect of enhanced expression of COL8A1 on lymphatic metastasis of hepatocellular carcinoma in mice

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Abbreviations: COL8A1, type VIII collagen one; FBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; RT-PCR, reverse transcription-polymerase chain reaction; MTT, 3-(4,5)-dimethylthiazol (-2-yl)-2,5 diphenyl-tetrazolium bromide

Key words: hepatocarcinoma, COL8A1, D-limonene, lymphatic metastasis

Abstract. The present study aimed to investigate the influence of COL8A1 expression on cell invasiveness, drug sensitivity and tumorigenicity of hepatocellular carcinoma Hepa1-6 cells with low metastatic potential. COL8A1-1-pEGFP-N2 and pEGFP-N2 were transfected into experimental and control group cells. The COL8A1 expression in transfected Hepa1-6 cells was analyzed with RT-PCR and western blot analysis. The invasive potential of transfected Hepa-6 cells was tested in invasion experiments in vitro and the tumorigenic ability of the transfected Hepa-6 cells was tested in mouse tumors in vivo. Hepa-6 cell proliferation and D-limonene sensitivity was analyzed using the MTT method. Expression of COL8A1 in the Hepa-6/COL8A1 group showed a significant increase when compared with the untransfected cells of the Hepa-6 control group and empty-plasmid transfected cells from the Hepa-6/mock control group. Enhanced COL8A1 expression increased cell proliferation and matrix adhesion ability via invasion and tumorigenesis in vivo while the sensitivity to D-limonene was concurrently inhibited. The expression of COL8A1 in hepatocarcinoma cells was correlated with increased tumor cell proliferation, invasion, in vivo tumorigenicity and reduced antitumor drug sensitivity, and may provide novel targets for tumor therapy.

Introduction

Hepatocarcinoma (liver cancer) is the third-leading cause of cancer-related mortality and the fifth most common malignancy worldwide (1). In China presently, hepatocarcinoma is the second major cause of cancer-related mortalities, with a mortality rate of 26.26 per 100,000 individuals (males, 37.55; and females, 14.45 per 100,000), accounting for 19.33% of all types of cancers. Accordingly, the estimated annual incidence of cases and mortalities from hepatocarcinoma are 360,000 and 350,000, respectively (2,3). Type VIII collagen, a non-fibrillar short-chain collagen, is a structural component of numerous extracellular matrices (4,5). It is highly expressed by vascular smooth muscle cells and is considered to be a key component in vascular remodeling (6,7). Type VIII collagen exists as a heterodimer composed of two distinct α-chains (COL8A1 and COL8A2), each with a molecular weight of approximately 60 kDa (8). The high expression level of COL8A1 in vascular endothelial cells and tumor cells has gained extensive attention (9,10). High expression of COL8A1 in tumor cells may be associated with tumor cell proliferation. The present study focused on the role of COL8A1 in the tumor metastasis of liver cancer, which may provide experimental basis for research and development of antitumor drugs.

Materials and methods

Cell culture. The mouse hepatocarcinoma cell line Hepa-6, which lacks metastatic potential in the lymph nodes (provided by the Cell Center of Peking Union Medical College, China) was cultured in DME medium (HyClone, Logan, UT, USA), supplemented with 10% FBS (HyClone), streptomycin 100 U/ml, penicillin 100 U/ml. The cells were incubated in a humidified atmosphere containing 5% CO2 at 37°C for 24 h. The cells were in logarithmic growth phase.

Cell transfection. COL8A1-1-pEGFP-N2 and pEGFP-N2 were transfected, into the experimental and control group cells, respectively. The specific steps were as follows. i) Hepa-6 cells were cultured in DME medium for 24 h, up to 40-80% confluency in cell fusion. ii) Carrier (6 μg) was dissolved in DME culture medium (without FBS and antibiotics) and when the volume reached 300 μl, 50 μl liposome reagent was added, mixed well and then kept at room
temperature for 5 min, and ultimately reaction complexes were formed. iii) Hepa-1-6 cells were prepared by adding 7 ml DMEM medium. Inclusion of such a complex containing the reaction of the test tube and 1 ml DMEM was mixed well and immediately transferred onto a dish. Cells were incubated in a humidified atmosphere containing 5% CO₂ at 37°C for 8 h by replacing the fluid for normal cell growth and continuing for an additional 48 h. The experimental group cells were cultured in G418 (800 μg/ml) selective medium, and the control group of untransfected Hepa-1-6 cells were cultured in G418 medium. Cells were cultured in the G418 (400 μg/ml) selective medium until the majority of the untransfected cells were dead in the control group and the transfected cells in the experimental group were cloned. The selective medium was replaced every 3 days for 20 days. A single clonal cell was picked for screening into 24-well plates using the cloning ring method together with cultured cells in G418 (400 μg/ml) selective medium.

RT-PCR analysis. For RT-PCR analysis of COL8A1 mRNA levels, total RNA was isolated from cells using TRizol (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized using an RT-PCR kit (Takara, Shiga, Japan) according to the manufacturer's instructions. The primer sequences were as follows; 5'-GCTGCTGGGAATACGTTCA-3'(F) and 5'-GGCCGTGAAGTCGTCAGAAC-3'(R) for COL8A1; 5'-GGCCGTGAAGTCGTCAGAAC-3'(F) and 5'-GCCACGATGCCCAGGAA-3'(R) for GAPDH. PCR analysis was performed under the following conditions: denaturation at 94°C for 1 min, and 30 cycles of denaturation for 20 sec at 97°C, annealing for 20 sec at 64°C and extension for 20 sec at 72°C. The amplified products were analyzed by agarose gel electrophoresis using 1.0% gel, followed by ethidium bromide staining.

Western blot analysis. Western blot analysis was performed to evaluate COL8A1 protein levels. The protein was harvested from cells using a 2X concentrated electrophoresis sample buffer (125 mmol/l Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 5% glycerol and 1% β-mercaptoethanol). Equal amounts of denatured proteins (35 µg) were resolved by 10% SDS-PAGE and then blotted onto PVDF membranes (Pall). Amounts of denatured proteins (35 µg) were resolved by 10% SDS-PAGE and then blotted onto PVDF membranes (Pall Corporation). After blocking for 2 h with phosphate-buffered saline containing 0.1% Tween 20 and 5% powdered skimmed milk, the blots were incubated with rabbit anti-mouse COL8A1 polyclonal antibodies (Abcam, Cambridge, UK; ab58776, 1:200 dilution) overnight in 5% powdered skimmed milk buffer, washed thrice with phosphate-buffered saline with 0.1% Tween 20 and then incubated with secondary antibody anti-rabbit-HRP (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; 1:3000 dilution). GAPDH antibody (Santa Cruz Biotechnology, Inc.; 1:200 dilution) was used as a control. All bands were detected using ECL western blot kit (Amersham Biosciences, Buckinghamshire, UK).

MTT assay. Hepa-1-6, Hepa-1-6/COL8A1 and Hepa-1-6/mock cells (1x10⁵) in 200 μl RPMI-1640 were seeded in duplicate into each well of 96-well culture plates, and 100 μl MTT (5 mg/ml, Sigma, St. Louis, MO, USA) was added at 24, 48, 72, 96 and 120 h. After a 4-h incubation at 37°C in 5% CO₂, 100 μl DMSO (Gibco, Carlsbad, CA, USA) was pipetted to solubilize the formazan product for 30 min at room temperature. The absorbancy of A570 was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). Growth rate (%) = A570(Hepa-1-6/COL8A1) ÷ A570(Hepa-1-6) x 100%.

In vitro ECM invasion assay. Cell invasion in vitro was demonstrated using 24-well Transwell units (Corning, NY, USA) with 8-µm pore size polycarbonate filter coated with ECMMatrix gel (Chemicon, Temecula, CA, USA) to form a continuous thin layer. Hepa-1-6, Hepa-1-6/COL8A1 and Hepa-1-6/mock cells (3x10⁵) were harvested in RPMI-1640 containing 1% FBS and added to the upper chamber. The lower chamber was filled with 500 μl RPMI-1640 containing 10% FBS. The cells were incubated for 24 h at 37°C with 5% CO₂. At the end of incubation, the cells on the upper surface of the filter were completely removed by wiping with a cotton swab. The filters were then fixed in methanol and were stained with Wright-Giemsa. The number of cells that had invaded the Matrigel and reached the lower surface of the filter were counted under a light microscope at a magnification of x200. Triplicate samples were acquired, and the data were expressed as the average cell number of 5 fields. Invasive cells were calculated and analyzed with the Image-Pro Plus 4.5 software (Media Cybernetics).

In vivo tumorigenicity analysis. A total of 60 C57L mice (provided by Dalian Medical University, Dalian, China) were randomly divided into three groups, each group having 20 mice. The logarithmic phase Hepa-1-6, Hepa-1-6/COL8A1 and Hepa-1-6/mock cells were injected subcutaneously in mice. The mice were sacrificed three weeks later. Swollen axillary lymph nodes, fixed with 4% formaldehyde from the 3 groups were generally compared for tumor weight and metastatic rates. Paraffin sections, hematoxylin and eosin staining and observation of tumor cells were performed under a microscope.

Drug sensitivity assay. To assess chemosensitivity to D-limonene (Sigma; Hepa-1-6, Hepa-1-6/COL8A1 and Hepa-1-6/mock cells (3x10⁵) cultured for 24 h, were incubated with various concentrations of D-limonene (0, 0.2, 0.4, 0.8 and 1.6 g/ml) for another 48 h. Then cells were treated with MTT as described previously and each group contained three wells. Cell survival rate (%) = A570(D-limonene) / A570(D-limonene) x 100.

Statistical analysis. SPSS 14.0 software (SPSS, Chicago, IL, USA) was used. Each assay was performed at least three times. The data are expressed as mean ± SD and the Student's t-test was used to determine the significance of differences in multiple comparisons. P<0.05 was considered to indicate a statistically significant result.

Results

cDNA transfection increases the expression of COL8A1. In order to verify whether stable expression of COL8A1 affects hepatocarcinoma tumors in mice, we transfected Hepa-1-6 cells that expressed COL8A1 at a low level with the constructed plasmid COL8A1-pEGFP-N2. After 3 weeks, we analyzed the stable transfected Hepa-1-6/COL8A1 cells by RT-PCR and western blot analysis. We set two negative control groups;
i) nontransfected Hepa1-6 cells; ii) empty plasmid-transfected Hepa1-6/mock cells, through which transfection of Hepa1-6 cells was observed. Following stable transfection, Hepa1-6/COL8A1 cells highly expressed COL8A1 and the other two control groups expressed COL8A1 at lower levels (Fig. 1). These results confirmed that it was feasible, reliable and effective to apply this method of stable transfection.

Enhanced COL8A1 expression increases Hepa1-6 cell proliferation in vitro. After enhancing the expression of the COL8A1 gene in Hepa1-6 cells, we determined the cell proliferation using MTT at 24, 48, 72, 96 and 120 h, and also set a control group of untransfected Hepa1-6 cells and another of empty plasmid-transfected Hepa1-6/mock cells. The results demonstrated that the growth rate curve of Hepa1-6/COL8A1 cells increased, while the growth rate curves of the control group exhibited a flat trend. Therefore, enhancing COL8A1 expression increased the proliferative ability of Hepa1-6 cells in vitro (Fig. 2).

Enhanced COL8A1 expression increases Hepa1-6/COL8A1 cell invasion in vitro. In order to verify whether the stable expression of COL8A1 plays a key role in Hepa1-6/COL8A1 cell invasion in vitro we compared the cell proliferation of Hepa1-6/COL8A1 cells of the experimental group with that of the two control groups: the untransfected cells of the Hepa1-6 control group and the empty plasmid-transfected cells of the Hepa1-6/mock control group (Fig. 3A). The number of cells that crossed the membrane was higher in the experimental group (21±3) (P<0.05; Fig. 3B). These results indicate that stable expression of COL8A1 is able to promote the in vitro cell invasion of Hepa1-6/COL8A1 cells and plays a key role in cancer metastasis.

Enhanced COL8A1 expression promotes the tumorigenicity of Hepa1-6 cells. The tumor formation experiment in vivo (Fig. 4A) showed that, compared with the control groups of untransfected Hepa1-6 cells and empty plasmid-transfected Hepa1-6 cells, the experimental group Hepa1-6/COL8A1 cells...
clearly exhibited tumor formation. These results indicate that the stable expression of COL8A1 promotes the tumorigenicity of Hepa1-6/COL8A1 cells.

**Increased COL8A1 expression reduces the sensitivity of Hepa1-6 cells to D-limonene treatment.** Compared with the control group of untransfected Hepa1-6 cells and empty plasmid-transfected Hepa1-6/mock cells, increased COL8A1 expression inhibited the sensitivity of Hepa1-6/COL8A1 cells to D-limonene treatment.

**Discussion**

One of the main purposes of cancer research is to identify the genetic factors that contribute to tumor formation and to elucidate the mechanism of tumor development. Therefore, certain specific gene proteins may be investigated due to their potential role as markers of tumor progression or as a treatment goal. It has been reported that at initial diagnosis, the cancer in over 60% of patients has progressed. Metastasis is a characteristic of malignant tumors and is the main cause of mortality in...
The stable expression of Hepa1-6/COL8A1 cells increased. The tumorigenicity in vivo trials demonstrated that the tumorigenicity of Hepa1-6/COL8A1 cells was clear (P<0.05). The stable expression of COL8A1 promoted the tumorigenicity of Hepa1-6/COL8A1 cells, which indicated that enhanced expression of COL8A1 affected Hepa1-6/COL8A1 cell in vivo tumorigenicity.

In conclusion, our results suggest that COL8A1 expression is closely related to tumor cell proliferation, invasion and tumorigenicity in vivo. COL8A1 overexpression inhibits drug sensitivity as determined by the effects of D-limonene on Hepa1-6/COL8A1 cells. COL8A1 expression may be one of the key mechanisms in the regulation of cytokines in tumor metastasis. Increased COL8A1 expression enhances tumorigenicity of the cells in the body, which provides new targets for the diagnosis and treatment of over 60% of the cancer patients lymph node metastasis. It also provides valuable experimental evidence and clues for the design of anticancer drugs.

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