Liver epithelial cells inhibit proliferation and invasiveness of hepatoma cells

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Abstract. Hepatocellular carcinoma (HCC) is a worldwide malignancy with poor prognosis. Liver progenitors or stem cells could be a potential therapy for HCC treatment since they migrate toward tumors. Rat liver epithelial (RLE) cells have both progenitor and stem cell-like properties. Therefore, our study elucidated the therapeutic effect of RLE cells in rat hepatoma cells. RLE cells were isolated from 10-day old rats and characterized for stem cell marker expression. RLE cells and rat hepatoma cells (H4-IIE-C3 cells) were co-cultured and divided into four groups with different ratios of RLE and hepatoma cells. Group A had only rat hepatoma cells as a control group. The ratios of rat hepatoma and RLE cells in group B, C and D were 5:1, 1:1 and 1:5, respectively. Effective inhibition of cell proliferation and migration was found in group D when compared to group A. There was a significant decrease in Bcl2 expression and increase in late apoptosis of rat hepatoma cells when adding more RLE cells. RLE cells reduced cell proliferation and migration of rat hepatoma cells. These results suggested that RLE cells could be used as a potential cell therapy.

Introduction

Hepatocellular carcinoma (HCC) is one of the leading malignancies worldwide, particularly in Asia (1-3). The treatment outcomes for those who are not candidates for resection or transplantation are usually miserable. Even after surgery, the long-term prognosis is generally unsatisfactory due to the high recurrence rate and the lack of effective systemic therapy (4,5). The benefit of the present target therapy remains limited (6-8). Therefore, it is urgent to develop a novel therapy for these patients.

Progenitor and/or stem cells could be potential therapeutic agents for HCC. Bone marrow-derived mesenchymal stem cells may inhibit hepatoma cell growth and their invasiveness (9-13). However, to obtain such cells from bone marrow is somewhat difficult in clinical practice. Therefore, it would be better to use liver progenitor cells to treat liver diseases.

Rat liver epithelial (RLE) cells from WB-F344 rats had the characteristics of progenitor or stem cells (14). They could reduce tumorigenity of hepatoma CBRH-7919 cells by TGFβ/Smad signaling. Peters et al found that co-culture of primary rat hepatocytes and RLE cells were able to enhance interleukin-6-induced acute phase protein responses (15). However, to use RLE cells as a weapon to treat HCC, two main points still need to be elucidated. One is whether these RLE cells inhibit tumor growth, and the other is whether they inhibit tumor invasiveness. Therefore, our study was focussed on the understanding of cell-to-cell interaction between RLE and HCC cells.

Materials and methods

Rat hepatoma cells. The rat hepatoma cell line H4-IIE-C3 was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Cells were cultured in the Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% penicillin/streptomycin (Gibco, Carlsbad, CA, USA) at 37°C in a humidified 5% CO2 incubator, and were routinely sub-cultured with 0.05% trypsin in phosphate-buffer at 80-90% confluence.

Isolation and culture of RLE cells. Pathogen-free Fisher (F344) rats were purchased from the National Laboratory Animal Center (Taipei, Taiwan). Rats were housed at Far Eastern Memorial Hospital. All animal study was performed in accordance within the guide for the care and use of laboratory animals and with the approval protocols of the Institutional Animal Care and Use Committee in the Far Eastern Memorial Hospital (FEMH; IACUC Approval No: 99-1-43-C1). Ten
day old Fisher F344 rats were used to isolate RLE cells. Liver pieces were incubated in a DMEM/F12 containing 10 mM HEPES (both from Gibco), 1 mg/ml type I V collagenase (Sigma, St. Louis, MO, USA) and 1% penicillin/streptomycin at 37°C for 20 min. RLE cells were plated on collagen I-coated culture dishes incubated at 37°C in incubator with 5% CO₂. Cells were grown in a stem cell medium containing DMEM/F12, 2% FBS, 10 mM HEPES, 0.1% ITS Premix (Corning, Corning, NY, USA), 1x10⁻³ M dexamethasone (Sigma), 10 ng/ml human stem cell factor (SCF; eBioscience, San Diego, CA, USA), 20 ng/ml epidermal growth factor (EGF) (Sigma, St. Louis, MO, USA) and penicillin/streptomycin.

Western blotting. RLE cell lysates were collected by a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, protease inhibitors, pH 7.5). Total protein (10 mg) was separated in SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Blots were blocked and incubated with primary antibodies against albumin, CK19 (Millipore, Billerica, MA, USA) and β-actin as a loading control (Sigma). The appropriate HRP-conjugated secondary antibodies were used and enhanced chemiluminescence (ECL) detection system (Millipore) were employed to visualize the proteins. Images were collected by ImageQuant™ LAS 4000 (GE Healthcare Life Sciences, UK).

Co-culture of hepatoma and RLE cells. Cell culture inserts (Millipore) were used to set-up a separated co-culture system. Rat hepatoma (5x10⁴) (H4-IIE-C3) cells were placed in the 24-well plate at the bottom and RLE cells were in the culture inserts. There were 4 groups for the co-culture systems. The control group (group A) only had H4-IIE-C3 cells without any RLE cells. In group B-D, different numbers of RLE cells: 1x10⁴, 5x10⁴ and 25x10⁴ were placed, respectively. The ratios of RLE to H4-IIE-C3 cells were 1:5; 1:1 and 5:1, respectively.

Cell proliferation assay. Rat hepatoma H4-IIE-C3 cells were separately co-cultured with different amount of RLE cells as above (group A-D). Rat hepatoma cells from each group were harvested after 24, 48, 72 and 96 h following the co-culture, respectively. The proliferation of rat hepatoma cells was measured by the WST-1 cell proliferation assay (Roche, Mannheim, Germany). The optical density (OD) values at 450/690 nm were measured by an ELISA reader (Bio-Rad, Hercules, CA, USA).

Flow cytometry. RLE cells were stained with stem cell marker Thy-1 PE (BD Pharmingen, San Jose, CA, USA). For apoptosis assay, rat hepatoma cells were stained with FITC/Annexin V apoptosis detection kit I (BD Pharmingen) after a 3-day co-culture. All procedures were followed by the manufacturer's instructions. The data were collected on a FACSCalibur (BD Biosciences) and analyzed by FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Migration assay. Transwell inserts (8-µm-pore) were used for the cell migration assay (Corning Inc., Tewksbury, CA, USA). In all groups, H4-IIE-C3 cells (5x10⁴) were placed on the cell inserts in a serum-free DMEM and RLE cells were cultured in stem cell medium in a 24-well plate. Group A had only H4-IIE-C3 cells without RLE cells. The cell number of RLE cells in group B-D was 1x10⁴, 5x10⁴ and 25x10⁴, respectively. The migrated rat hepatoma cells were collected after 24 h post-incubation at 37°C. The migrated cells were fixed with 10% formaldehyde and washed with phosphate-buffered saline (PBS). The cells were then stained with 0.4% Giemsa (Sigma) for 2 h and washed with sterile ddH₂O. Images (magnification, x100) were collected under a Leica microscope (Leica Microsystems, Wetzlar, Germany).

RNA isolation and quantitative real-time PCR. Total RNA was isolated from rat hepatoma cells H4-IIE-C3 by the innuPREP RNA Mini kit (Analytik Jena, Jena, Germany) according to the manufacturer's protocol. The total RNA was reversely transcribed to cDNA by a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). The mRNA expression was analyzed by a real-time PCR Roche LightCycler 480 (Roche Applied Science, Mannheim, Germany). For real-time PCR, procedures were as follows: hot start at 95°C for 1 min, followed by 45 cycles of denaturing at 95°C for 10 sec, annealing at 58°C for 5 sec and extension at 72°C for 20 sec. PCR products were detected using 2% agarose gel to confirm the expected sizes. All primer sequences for quantitative real-time PCR analysis were: Bcl2 forward, 5'-CGA CTT TGC AGA TGT CCA-3' and Bcl2 reverse, 5'-ATG CCG GTT CAG GTA CTG AC-3'; Bax forward, 5'-GAG AGG ATG GCT GGG GAG AC-3' and Bax reverse, 5'-GAG AGG ATG GCT GGG GAG AC-3'. GAPDH forward, 5'-TGA GTG AGG CAC CAA CTG AG-3' and GAPDH reverse, 5'-TTG CAC CAC CCA CTG AG-3' and GAPDH reverse, 5'-CTT CAC CAC CTT CTT CAT G-3'. Gene expression was analyzed after normalization to control gene GAPDH.

Statistical analysis. Comparisons among groups were performed using SPSS (SPSS, Inc., Chicago, IL, USA). All the data are reported as mean ± SD. Comparisons between different groups for each point were performed using the one-way analysis of variance (ANOVA; and Kruskal-Wallis test), and multivariate analysis. All tests were two-tailed, and p<0.05 was considered to indicate a statistically significant result.

Results

Characterization of RLE cells. Isolated RLE cells started to form a colony within 2 days and the cells were confluent after a 5-day culture (Fig. 1A and B). The morphology of RLE cells changed after a few passages, notably when comparing the 1st and 4th passage (Fig. 1C and D). Cells from the 1st passage were round, whereas the 4th passage cells became fibroblast-like, which suggested that cells became unhealthy after the 4th generation passage. Therefore, RLE cells only from the 2nd and 3rd passages were used in the present study.

In addition, 75% of RLE cells expressed stem cell marker Thy-1 (Fig. 2A). Notably, stem cell markers CK19 and EpCAM as well as hepatocyte marker albumin were detected in RLE cells (Fig. 2B).
RLE cells reduce cell proliferation of rat hepatoma cells. There was a decreased tendency of cell proliferation in rat hepatoma cells when the RLE cells increased (Fig. 3). However, there was no difference between group A and D; B and C; or C and D. A significant difference between group B and D (p=0.049) at day 4 was found, which showed that an increased cell number of RLE cells reduced the cell proliferation in rat hepatoma cells.

RLE cells increased apoptosis of rat hepatoma cells. There were no differences in early apoptosis (Annexin V+/PI-) among the groups. Group A (hepatoma cells only) had 3% early apoptotic cells and 16% late apoptotic cells. However, the percentages of late apoptotic cells (Annexin V+/PI+) increased among the other three groups, from 10% (group B), 14% (group C) to 16% (group D) (Fig. 4). This shows that the apoptotic rates of rat hepatoma cells increased by adding more RLE cells.
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RLE cells reduced cell migration of rat hepatoma cells. The migration of rat hepatoma was examined at the early time point (day 2) since RLE cells could reduce cell proliferation at the late time point (day 4). There was a significant decrease of the number of the migrated rat hepatoma cells in comparison between group A and D (p=0.029) (Fig. 5). In addition, there was a decreased trend of migrated rat hepatoma cells when RLE cell number increased. Therefore, RLE cells inhibited the migration of rat hepatoma cells.

Decreased Bcl2 expression in rat hepatoma cells with increasing RLE cells. When comparing group A (rat hepatoma cells only) to group D (rat hepatoma cells: RLE cells=1:5), there was a significant decrease of the survival gene Bcl2 (p=0.005) (Fig. 6A). There seemed to be an increase of apoptotic gene Bax expression in group D. However, it was not statistically significant (Fig. 6B). It suggested that increasing RLE cells can reduce the survival of rat hepatoma cells.

Discussion

Rat liver epithelial (RLE) cells isolated from adult and newborn rats have similar oval cell-like properties (16). In our study, RLE cells were isolated from new-born rats and they...
Figure 5. Reduced cell migration of rat hepatoma cells by RLE cells. Rat hepatoma cells (5x10^4) were seeded in cell inserts and different amount of RLE cells were seeded into 24-well plates. Group A had only rat hepatoma cells. The ratios of H4-IIE-C3:RLE were 5:1, 1:1 and 1:5 in group B-D. The y-axis represented the migrated rat hepatoma cells counted under 5 different fields (at least 3 independent experiments; *p<0.05).

Figure 6. Altered apoptotic gene expression in rat hepatoma cells with increasing RLE cells. Group A had only rat hepatoma cells. The ratios of H4-IIE-C3:RLE were 5:1, 1:1 and 1:5 in group B-D. The mRNA from rat hepatoma cells were isolated and subjected to quantitative PCR for gene expression in Bcl2 in (A) and Bax in (B); ( *p<0.05; **p<0.01).
could express progenitor cell or stem cell makers such as Thy-1, CK19, EpCAM and albumin. Thy-1 and CK19 are also expressed in human fetal livers (17). Hepatic progenitor cells express the markers EpCAM, CK7, CK19, α-fetoprotein (AFP) and Thy-1 (18-20). It suggests that RLE cells have similar properties and characteristics as hepatocyte progenitor cells.

Two important points were found in the present study. First, RLE cells reduced the cell proliferation and survival of hepatoma cells. Second, RLE cells could also inhibit the migration of tumor cells. Both effects significantly enhanced when RLE cell number was increased. RLE cells induced apoptosis and reduced the survival of rat hepatoma cells due to downregulation in Bcl2. Bcl2 is a survival factor and highly expressed in hepatocellular carcinoma (HCC) cells. Therefore, targeting Bcl2 could induce cell death in tumors (21). It is also true that hepatocyte precursors increased apoptosis of rat hepatoma cells by decreasing Bcl2 and c-Myc (14).

In conclusion, RLE cells were able to inhibit proliferation and invasiveness of rat hepatoma cells. Therefore, RLE cells have a high potential for cell therapy in hepatoma cells.

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Table I. Mesenchymal stem cells either suppress or promote tumor growth.

In conclusion, RLE cells were able to inhibit proliferation and invasiveness of rat hepatoma cells. Therefore, RLE cells have a high potential for cell therapy in hepatoma cells.

Acknowledgements

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