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% CO_2 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

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Abbreviations: HCC, hepatocellular carcinoma; RLE, rat liver epithelial; AFP, α-fetoprotein; EMT, epithelial-mesenchymal transition; iPS, induced pluripotent stem; NSC, neural stem cells; MSC, mesenchymal stem cells; PI, propidium iodide

Key words: rat liver epithelial cells, cell therapy, hepatocellular carcinoma, Bcl2, apoptosis

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 IV 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 humidity incubator with 5% CO_2. Cells were grown in a stem cell medium containing DMEM/F12, 2% FBS, 10 mM HEPES, 0.1% ITS Premix (Corning, Corning, NY, USA), $1x10^{-7}$ 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 (10712-1-AP), EpCAM (21050-1-AP) (both from Proteintech, Chicago, IL, USA) and β-actin as a loading control (Sigma). The appropriate HRP-conjugated secondary antibodies were used and enhanced chemiluminescence (ECL) detection system (Millipore) was 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 ($5x10^4$) (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: $1x10^4$, $5x10^4$ and $25x10^4$ 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 ($5x10^4$) 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 $1x10^4$, $5x10^4$ and $25x10^4$, respectively. The migrated rat hepatoma cells were evaluated 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 ddH_2O. 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 manufacture's protocol. The total RNA was reversely transcripted 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 CTC AG-3'; *Bax* forward, 5'-GAG AGG ATG GCT GGG GAG AC-3' and *Bax* reverse, 5'-TGA GTG AGG CAG TGA GGA CT-3'; *GAPDH* forward, 5'-CAC CAC CAA CTG CTT AG-3' and *GAPDH* reverse, 5'-CTT CAC CAC CTT CTT GAT 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).

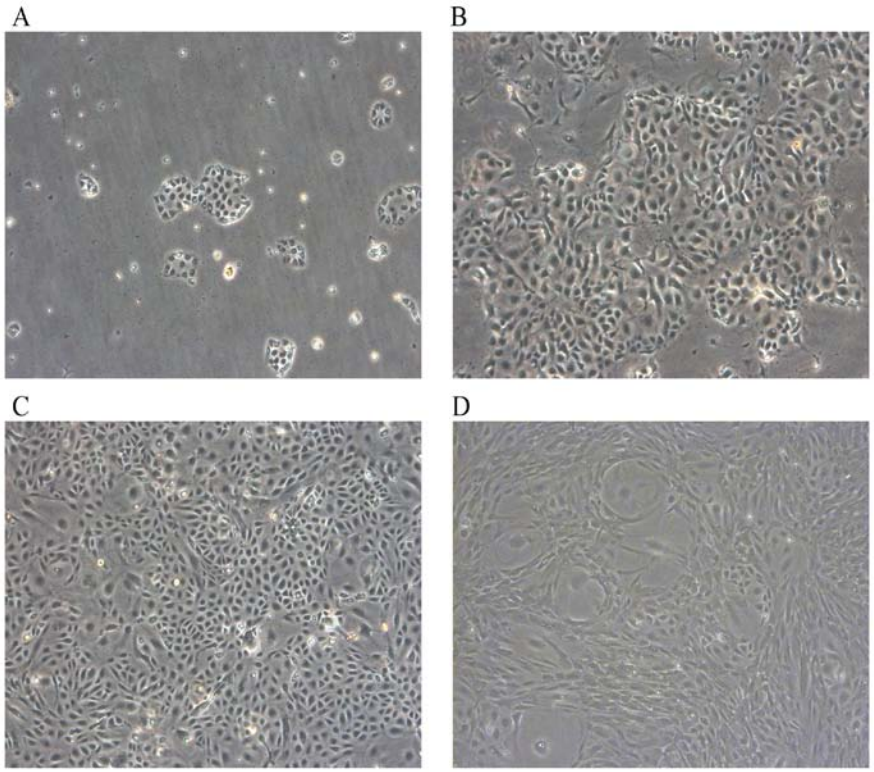

Figure 1. Cell morphology of rat liver epithelial cells. (A) Two day culture. (B) Five day culture. (C) First passage. (D) Fourth passage. Magnification, objective x50.

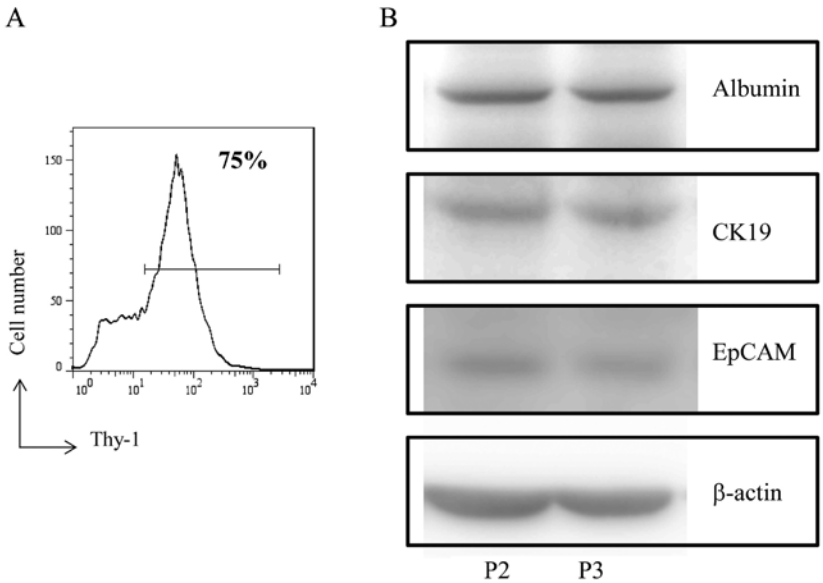

Figure 2. Characterization of rat liver epithelial cells. (A) Thy-1 expression in RLE cells. Cells were stained with anti-Thy-1 PE and analyzed by flow cytometry. (B) Protein expression of AFP, CK19, albumin and EpCAM in RLE cells were analyzed by western blotting and β-actin as a loading control. P2, 2nd passage; P3, 3rd passage.

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 apoptosis 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.

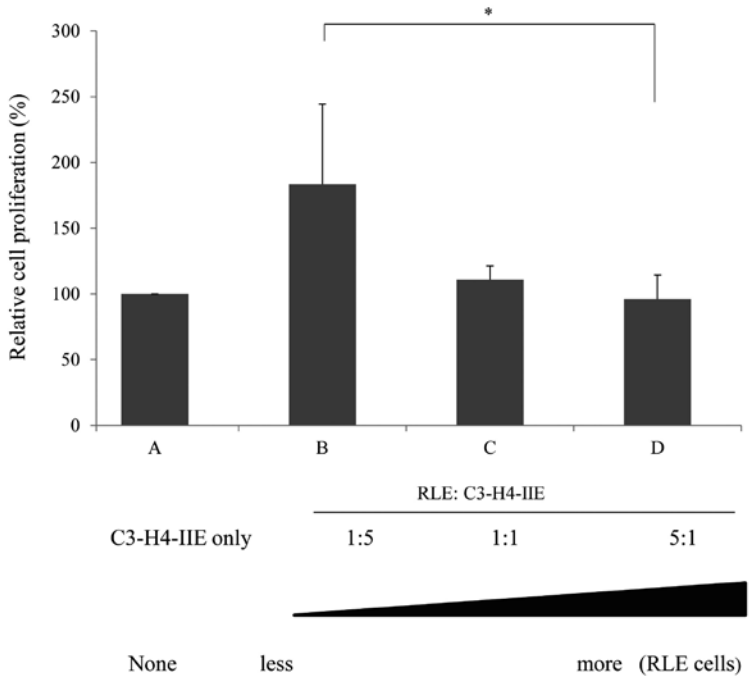

Figure 3. Reduced cell proliferation of rat hepatoma cells by RLE cells. Cell proliferation of rat hepatoma cells were detected by WST-1 assay at day 4. All the groups had the same cell number of rat hepatoma cells. Group A had only rat hepatoma cells. The ratio of hepatoma cells to RLE cells in group B-D was 5:1, 1:1 and 1:5, respectively; (*p<0.05).

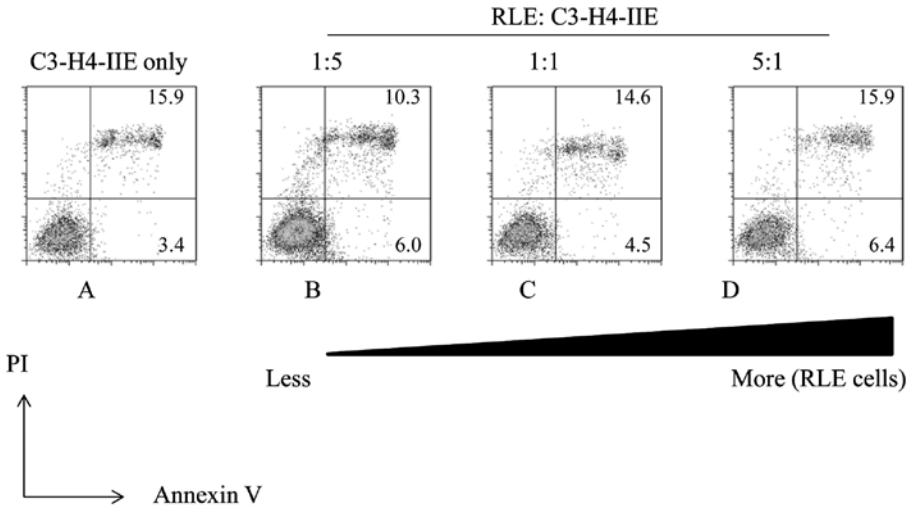

Figure 4. Increased late apoptosis of rat hepatoma cells by RLE cells. Rat hepatoma cells were stained with Annexin V and propidium iodide (PI) to detect apoptotic cells by flow cytometry. The number in the right upper quadrant (Annexin V^+/PI^+) represented the percentages of late apoptotic cells whereas the number in right bottom quadrant (Annexin V^+/PI^-) represented the percentages of early apoptotic cells.

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 new-born rats have similar oval cell-like properties (16). In our study, RLE cells were isolated from new-born rats and they

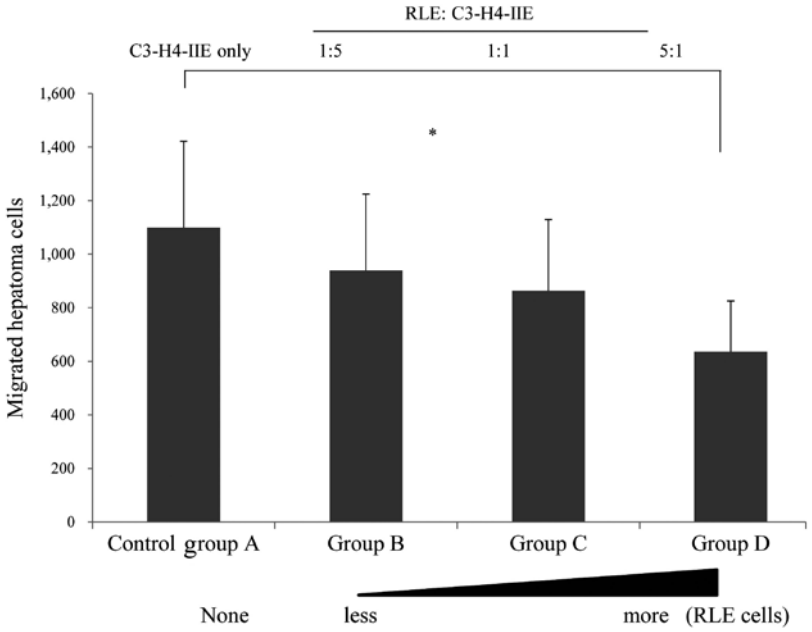

Figure 5. Reduced cell migration of rat hepatoma cells by RLE cells. Rat hepatoma cells ($5x10^4$) were seeded in cell inserts and different amount of RLE cells were seeded into 24-well plates. Group A had only rat hepatoma cells. The ratios of H4-IIE-C3:RLE were 5:1, 1:1 and 1:5 in group B-D. The y-axis represented the migrated rat hepatoma cells counted under 5 different fields (at least 3 independent experiments; $^{*}p<0.05$).

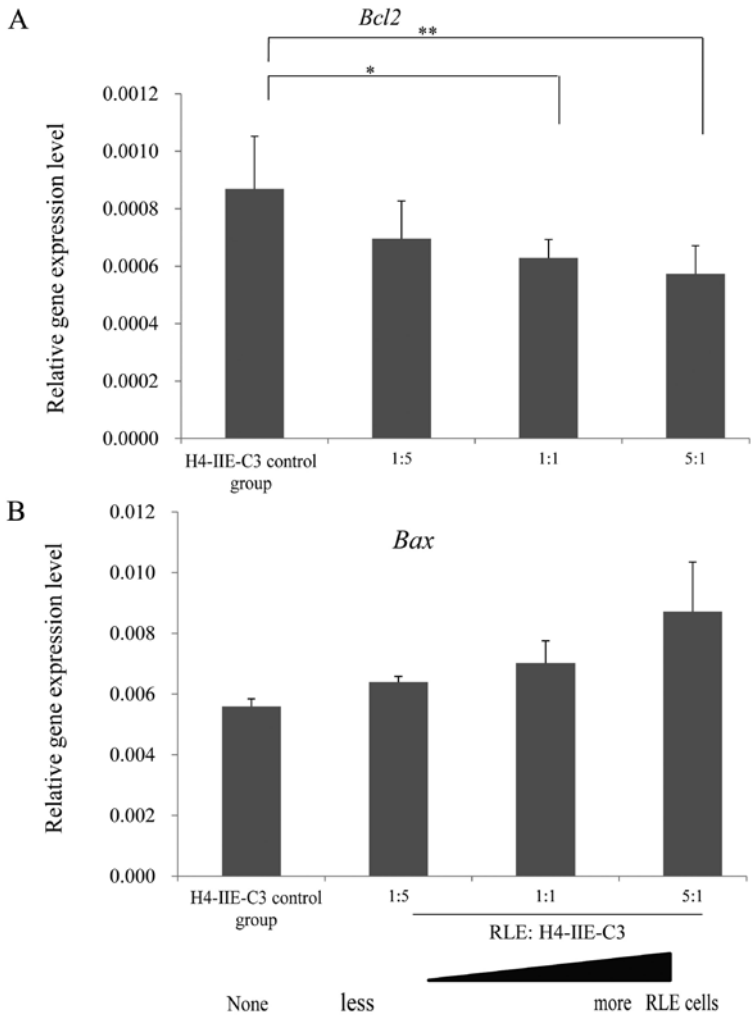

Figure 6. Altered apoptotic gene expression in rat hepatoma cells with increasing RLE cells. Group A had only rat hepatoma cells. The ratios of H4-IIE-C3: RLE were 5:1, 1:1 and 1:5 in group B-D. The mRNA from rat hepatoma cells were isolated and subjected to quantitative PCR for gene expression in *Bcl2* in (A) and *Bax* in (B); ($^{*}p<0.05$; $^{**}p<0.01$).

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

Author	Neoplasm	Effect	Refs.
Ho *et al*	Glioma	Suppression	10
Lu *et al*	Murine hepatoma H22 Lymphoma (YAC-1 and EL-4) Rat insulinoma INS-1 cell line	Inhibition	29
Ramasamy *et al*	Tumor cell line of hematopoietic (BV173)	Inhibition	30
Khakoo *et al*	Kaposi's sarcoma	Inhibition	31
Elzaouk *et al*	Mouse melanoma	Antitumor, *IL-12*	32
Li *et al*	HCC	Enhanced tumor growth but inhibition invasiveness and metastasis	33
Wang *et al*	Esophageal cancer	Declining tumorigenicity	34
Long *et al*	HepG2 cell line	Growth inhibitory	11
Bruno *et al*	HepG2 hepatoma Kaposi's sarcoma Skov-3 ovarian tumor cell lines	Inhibit *in vitro* cell growth and survival of different tumor cell lines and *in vivo* tumor growth	12
Qiao *et al*	H7402 and HepG2 human liver cell line	Inhibit proliferation, colony-forming ability and oncogene expression both *in vitro* and *in vivo*	35
Ganta *et al*	Breast cancer	Antiproliferative	36
Zhu *et al*	F6 tumor cell line SW480 tumor cell line	Favor growth	37
Djouad *et al*	Melanoma tumor cells	Favor growth	38
Jung *et al*	Prostate cancer	Promote metastasis	39
Karnoub *et al*	Breast cancer	Promote metastasis	40

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 addition, RLE cells were able to reduce the migration of rat hepatoma cells. Tumor migration is one characteristic of cancer invasiveness (22,23). It could come from cytokines or chemokines made by RLE cells since the co-culture system was used without cell-to-cell contact. Previous study showed cytokine IL-6 was found to increase fibrinogen and decrease albumin secretion when co-culturing epithelial cells with hepatocytes (15). It is possible that IL-6 participated in the reduction of cell migration. TGFβ and its receptors (TBRI and TBRII) are involved in tumor cell invasion through epithelial-mesenchymal transition (EMT). Therefore, increased TGFβ receptor and Smad expression was found in rat hepatoma cells when increasing hepatocytic precursor cells (14).

In other studies, rat liver stem cells may selectively migrate to rat HCC due to chemoattractants or factors such as VEGF, PDGF, TGF-β, MCP-1, IL-8, TNF-α, IL-1β, IL-6, SDF-1 or HGF (24-26). Therefore, hepatocyte progenitor cells could be used for cell therapy in HCC (27).

Stem cells for cancer therapy have been reported. Human-induced pluripotent stem cells (iPS) which can differentiate into neural stem cells (NSC) may be used as a cellular delivery vehicle for cancer gene therapy (28). It remains complex that mesenchymal stem cells (MSCs) either promote or inhibit different tumors (Table I). MSCs derived from bone marrow have characteristics in tumor suppression (10-12,29-36). On the contrary, some found bone marrow-derived MSCs promote tumor growth (37-40). However, MSCs could act as a therapeutic tool and gene carrier for liver fibrosis and HCC (41,42). Whether stem cells or progenitor cells inhibit or promote tumor growth remains controversial, depending upon the stem cell origin and the difference among neoplasms.

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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