Effect of human mesenchymal stem cells on hepatoma cell line

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Abstract

Human mesenchymal stem cells (hMSCs) are mostly studied for their potential clinical use. Little is known about the underlying molecular mechanisms that link hMSCs to the targeted inhibition of tumor cells. The present study was conducted to evaluate the tumor suppressive effects of human mesenchymal stem cells (hMSCs) on human hepatoma cell line (HepG2) and their signaling mechanisms. To fulfill this objective, the influence of hMSCs on genes concerned with apoptosis, mitogenesis as well as on the proliferation of HepG2 cell line was investigated using either hMSCs-conditioned or using hMSCs and HepG2 co-culture conditioned media. Cell survival was evaluated using cell proliferation (MTT) assay kit. Gene expression of survivin, proliferating cell nuclear antigen (PCNA), β-Catenin, telomerase and VEGF was assessed by real time reverse transcription-polymerase chain reaction (RT-PCR). HpG2 cells cultured with either hMSCs-conditioned media or with hMSCs&HepG2 co-culture conditioned media showed decreased proliferation and decreased expression of survivin, PCNA, β-Catenin and telomerase. However, both media had increased expression of VEGF. Treatment of HepG2 cells by either hMSCs conditioned media or by hMSCs and HepG2 co-culture condition media led to a significant decrease in cell proliferation and down regulation of genes concerned with antiapoptosis, mitogenesis, and cell proliferation. This indicates that hMSCs can suppress tumorigenesis through factors produced in their conditioned media.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common solid tumors worldwide;[1] it is the fifth most frequently fatal human cancer 2. Surgical resection and liver transplantation are the two curative treatments for HCC, but are applicable to only a small proportion of patients with early tumors.[3,4] Currently, there is no proven effective systemic chemotherapy for HCC, whereas alternative treatment strategies such as transcatheter arterial chemoembolization, percutaneous intratumoral ethanol injection, and radiofrequency ablation are mainly for palliation and are applicable only to patients with tumors localized in the liver.[5]

Mesenchymal stem cells are known as multipotent and exhibit the potential for differentiation into different cells/tissue lineages, including cartilage, bone, adipose tissue, tendon, and ligament.[6] MSCs possess numerous properties that might make them an attractive choice as a cell-mediated gene therapy in human malignancies.[7,8]

The inhibition of tumor growth by MSCs has been observed in different types of animal models. In experimental models of Lewis lung carcinoma and B16 melanoma (mouse melanoma cell line) Maestroni *et al*., first reported that the co-injection of mouse MSCs with tumor cells inhibited primary tumor growth. Although the factors mediating the antitumor activity of MSCs were not identified by the authors, data from that study suggested that they were distinct from inflammatory cytokines.[9] The antiproliferative action of MSCs was also reported in a model of colon carcinogenesis in rats, in which the co-injection of MSCs with tumor cells in a gelatin matrix implanted subcutaneously led to growth inhibition. In addition, the co- injection of both cells triggered a more pronounced infiltration of monocytes and granulocytes.[10]

MSCs have been shown to express transgenes efficiently and for an extended period without any defect in their stem cell properties.[11] The stem cell microenvironment has an essential role in preventing carcinogenesis by providing signals to inhibit proliferation and to promote differentiation.[12]

Beckermann *et al*. demonstrated that high VEGF was produced by MSCs in pancreatic carcinoma, which was further enhanced by hypoxia. Therefore, secretion of VEGF may be the crucial factor determining the angiogenic potential of MSCs.[13]

Human MSCs home to sites of Kaposi's sarcoma, and potently inhibit tumor growth *in vivo* by downregulating Akt activity in tumor cells that are cultured with hMSCs prior to transplantation in animal tumor models.[7] Abdel Aziz *et al*. reported that hMSCs were able to suppress hepatocarcinogensis in rats, an effect that may involve the Wnt signaling pathway.[14]

The concept of MSCs has been broadened to include the secretion of biologically active molecules that exert beneficial effects on other cells.[15] So, MSCs may have a therapeutic effect even if they do not engraft or differentiate into tissue-specific cells, which significantly increase the range of MSCs therapeutic applications.

In this study, we investigated the effect of hMSCs on human hepatoma Cell line (HepG2) using hMSCs conditioned media and hMSCs and HepG2 co-culture conditioned media.

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Materials and Methods

Isolation and cultureof human mesenchymal stem cells

Under general anesthesia, about 10 mL of bone marrow was drawn from the iliac crest in a syringe containing 1500 U of heparin. Bone marrow was obtained from normal adult donors after informed consent and under a protocol approved by an Institutional Review Board. The isolation of MSCs was performed using the methods of Johnstone *et al*.[16] and Kadiyala *et al*.[17]. In brief, the bone marrow aspirate was layered onto Histopaque-1077 (Sigma, St. Louis, MO, USA) and centrifuged at 400 g for 30 min. The collected buffy coat was mixed with 20 mL of Dulbecco's phosphate-buffered saline (DPBS) and centrifuged at 300 g for 5 min. The supernatant was discarded and the cells were washed two more times with DPBS. After determination of cell viability and the number of viable cells by trypan blue staining, the cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS; USDA, Gibco, Grand Island, NY, USA) and antibiotics (penicillin

10,000 U/mL, streptomycin 10,000 lg /mL, amphotericin B 25 µg/mL). The nucleated cells were plated in tissue culture flask at 2.5×10^5/cm^2 and incubated at 37°C in a humidified atmosphere containing 5% CO_2. On day 4 of culture, the non-adherent cells were removed along with the change of medium. On day 14, the adherent colonies of cells were trypsinized, and counted. Cells were identified as being MSCs by their morphology, adherence, and their power to differentiate into osteocytes[18] and neurocytes.[19] Differentiation into osteocytes was achieved by adding 1-1000 nM dexamethasone, 0.25 mM ascorbic acid, and 1-10 mM beta-glycerophosphate to the medium. Kinetic quantitative determination of alkaline phosphatase (ALP) was carried out in the medium of differentiated cells using a commercial kit provided by Stanbio laboratory (Boerne, TX, USA). Differentiation into neurocytes was achieved by adding beta-mercaptoethanol, dimethyl sulfoxide, and conditioned medium for neuron induction. Differentiation was confirmed by detection of nerve growth factor (NGF) gene expression in cell homogenate. MSCs from passage 4 were used in this study upon reaching 70-80% confluence.[20]

Cultures of human hepatoma (HepG2) cells

Human Hepatoma (HepG2) cells were obtained from ATCC (American Type Culture Collection) and were grown in a sterile 50 cm^2 tissue culture flask in complete medium containing DMEM supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) in 95% air/5% CO_2 at 37°C. Cells were cultured to 100% confluence.[21] Cells from passage 14 were used in this study.

Co-culture of mesenchymal stem cells and HepG2 cells

The growth medium of cultured HepG2 cells was removed and adherent cells were washed twice with 1x PBS and detached by incubation of cells with trypsin (2.5 g/L)/EDTA (1 g/L) for 4-5 min at 37°C. Cells were centrifuged at 1000 rpm for 5-10 min, at 17°C. Cells were resuspended in DMEM and the cell suspension was added to the cultured MSCs.[21] So, the co-culture medium was composed of DMEM (Sigma) supplemented with 10% fetal bovine serum (FBS; USDA, Gibco, Grand Island, NY, USA) and antibiotics penicillin 10,000 U/mL, streptomycin 10 000 l g/mL, amphotericin B 25 µg mL. The ratio of MSCs: HepG2 cells was 1:1 and cells were co-cultured for 72 h.

At that time, the media were collected from the flasks and were used as hMSCs and HepG2 co-culture conditioned media.

Treatment of HepG2 with mesenchymal stem cells conditioned media

Human MSCs were cultured as described above. At the proper time of experiment (4th passage of MSCs) the media were collected from the flasks leaving the adherent MSCs. These media served as MSC conditioned media. HepG2 cells were treated with a mixture of complete medium containing DMEM supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) and hMSCs conditioned medium (1:1) for 48-96 h, and the culture medium was replaced every 24 h.

Treatment of HepG2 with co-culture conditioned media

HepG2 cells were treated with co-culture conditioned media for 48-96 hours, and the culture media were replaced every 24 h.

Cell proliferation assay

Cell proliferation of HepG2, HepG2 treated with MSC conditioned media and HepG2 treated with co-culture conditioned media n was determined using the MTT (3-[4, 5-dimethylthiazol-2-yl]-2.5-diphenyl tetrazolium bromide) cell proliferation kit (Trevigen Inc., Gaithersburg, MD, USA) as per manufacturer's protocol. Briefly, cells were plated in 96-well tissue culture plates in a range of $103\text{-}10^5$ cells/well in a final volume of 100 µL of medium and were allowed to attach overnight. The MTT reagent is added (10 µL per well) and the plate is incubated for 2 to 12 h to allow for intracellular reduction of the soluble yellow MTT to the insoluble purple formazan dye. Detergent reagent is added to each well to solubilize the formazan dye prior to measuring the absorbance of each sample in a microplate reader at 550-600 nm. Six wells were used for each group. Cell proliferation was assessed as the percentage of cell proliferation compared to untreated HepG2 as control cells.

Real-time quantitative analysis for Survivin, PCNA, β-Catenin, Telomerase and VEGF

Total RNA was isolated from the HepG2 cells using RNeasy purification reagent (Qiagen, Valencia, CA, USA). cDNA was generated from 5 µg of total RNA extracted with 1 µL (20 pmol) antisense primer and 0.8 µL superscript AMV reverse transcriptase for 60 min at 37°C. The relative abundance of mRNA species was assessed using the SYBR® Green method on an ABI prism 7700 sequence detector system (Applied Biosystems, Foster City, CA, USA). PCR primers were designed with Gene Runner Software (Hasting Software, Inc., Hasting, NY) from RNA sequences from GenBank (Table 1). All primer sets had a calculated annealing temperature of 60°. Quantitative RT-PCR was performed in duplicate in a 25 µL reaction volume consisting of 2X SYBR Green PCR Master Mix (Applied Biosystems), 900 nM of each primer and 2-3 µL of cDNA. Amplification conditions were 2 min at 50°, 10 min at 95° and 40 cycles of denaturation for 15 s and annealing/extension at 60° for 10 min. Data from real-time assays were calculated using the v1·7 Sequence Detection Software from PE Biosystems (Foster City, CA, USA). Relative expression of Survivin, PCNA, β-Catenin, Telomerase and VEGF mRNA was calculated using the comparative Ct method as previously described. All values were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene and reported as fold change over background levels detected in HepG2 group.

Table 1. Sequence of the primers used for real-time PCR.

Gene	Sequence
PCNA	Forward: 5'-AAA AAA GAC TAT GAA GTG GGT AGG-3' Reverse: 5'-CTG TTT CTA CAG TGC ATT GTA TAC G-3'. GenBank Accession: G08890
Survivin	Forward: 5'-ATG GCA CGG CGC ACT TT-3' Reverse: 5'-TCC ACT GCC CCA CTG AGA A-3' GenBank accession: NM 001168
β -catenin	Forward: 5'-GGA AGG TGG GAT TTT TGG TT-3' Reverse: 5'-TCC TCT TCT GCT CTT TTC TTG G-3'. UniGene Hs.166011
VEGF	Forward: 5'-CTG CTG TCT TGG GTG CAT TG-3' Reverse: 5'-TTC ACA TTT GTT GTG CTG TAG-3'. UniGeneHs.73793
Telomerase	Forward: 5'-ACT CGA CAC CGT GTC ACC TA-3' Reverse: 5'-GTG ACA GGG CTG CTG GTG TC-3'. UniGene Hs.492203
GAPDH	Forward: 5'- GAAGGTGAAGGTCGGAGTCA-3' Reverse: 5'- GAAGATGGTGATGGGATTTC-3'. UniSTS:273246

Statistical analysis

Data are expressed as mean ± SD. Kruskal-Wallis test is used as a non-parametric test for analysis of variance. Multiple comparisons between the groups were conducted using Dwass-Steel-Critchlow-Fligner test. Statistical analysis was performed using KyPlot Statistics software version 5.0 developed by Kyens Lab. Inc. Japan (www.kyenslab.com). Results were considered significant at P<0.05.

Results

The present study was conducted in vitro to assess the effect of conditioned media of human MSCs on HepG2 cells. The cultured cells were divided into 3 groups: HepG2 cells as control cells, HepG2 cells treated with MSC conditioned medium and HepG2 cells treated with co-culture conditioned medium.

Cellular proliferation

According to MTT test (Figure 1), differences in the viability of HepG2 cells in the presence of MSCs conditioned media and co-culture conditioned media were observed. Viability of both HepG2 cells treated with MSCs conditioned media and HepG2 cells treated with co-culture conditioned media was decreased significantly (P<0.001) as compared to untreated HepG2 cells. There was no statistical significant difference in viability between HepG2 cells treated with MSC conditioned medium and HepG2 cells treated with co-culture conditioned medium.

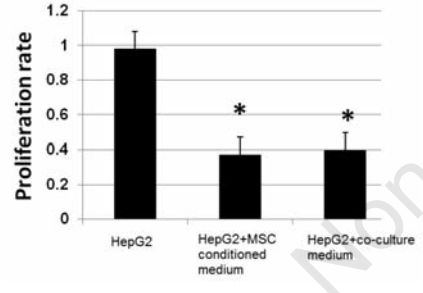

Figure 1. Inhibition of HepG2 cell proliferation. *P<0.001 *vs.* HePG2 (control).

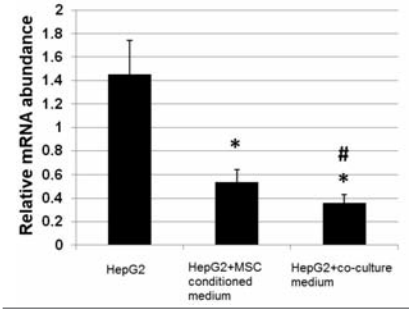

Figure 2. Relative mRNA abundance for Survivin in different groups. (*P<0.001 vs. control; #P<0.05 versus HepG2+MSC conditioned medium).

Quantitative real time PCR

Quantitative gene expression of survivin, PCNA, β-Catenin, telomerase and VEGF genes was detected in HepG2 cells by real time PCR.

Treatment of HepG2 cells with MSC conditioned medium and co-culture conditioned medium resulted in a significant downregulation of survivin (P<0.001) (Figure 2), PCNA (P<0.001) (Figure 3), β-Catenin (P<0.001) (Figure 4) and telomerase (P<0.001) (Figure 5) in comparison to untreated HepG2 cells. There was no statistically significant difference in gene expression between HepG2 cells treated with MSC conditioned media and HepG2 cells treated with co-culture-conditioned media in telomerase and β-Catenin. However, gene expression of survivin was more downregulated using co-culture conditioned medium than MSC conditioned medium (P<0.05).

PCNA gene expression was more downregulated using MSC-conditioned medium than co-culture conditioned medium (P<0.05).

As regards VEGF, there was statistically significant increase in its gene expression after treatment with MSC and co-culture conditioned media. (P<0.05) (Figure 6). However, there was no statistically significant difference between HepG2 cells treated with MSC conditioned media and HepG2 cells treated with co-culture-conditioned media.

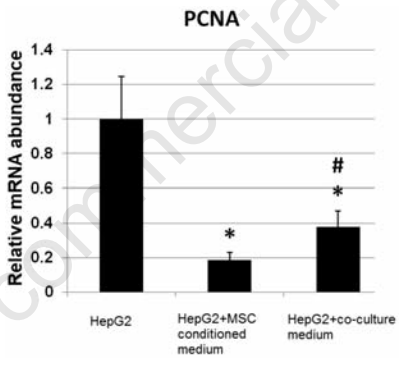

Figure 3. Relative mRNA abundance for PCNA in different groups. (*P<0.001 *vs.* control; #P<0.05 *vs.* HepG2+MSC conditioned medium).

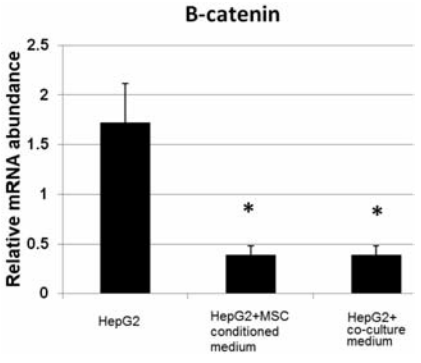

Figure 4. Relative mRNA abundance for β Catenin in different groups. (*P<0.001 *vs.* control).

Discussion

MSCs can represent a hope for cancer therapies, but a thorough evaluation of their potential risk is a prerequired step.[22] The present study was conducted to assess the effect of conditioned media of hMSCs and co-culture conditioned media on the progression of Hepatoma cell line (HepG2 cells). It was found that MSC conditioned media and co-culture conditioned media decreased proliferation rate of HepG2 cells.

The present work also showed that the expression of PCNA, survivin and β-Catenin

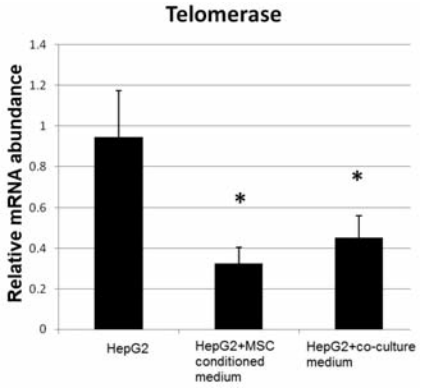

Figure 5. Relative mRNA abundance of telomearse in different groups. (*P<0.001 *vs* control).

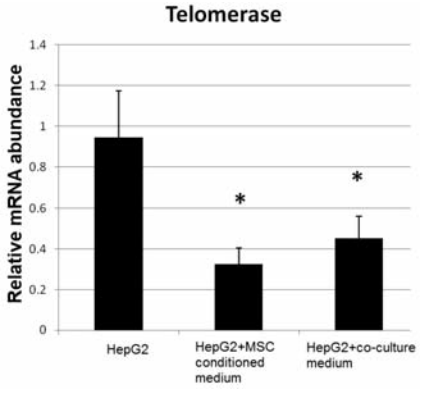

Figure 6. Relative mRNA abundance for VEGF in different groups. (*P<0.05 *vs.* control).

genes by HepG2 cells was significantly reduced by addition of hMSCs conditioned media and co-culture conditioned media. These results coincide with our previous work *in vivo* in hepatoma model.[14] The authors stated that the use of MSCs in diethylnitrosamine-induced carcinogenesis has tumor suppressive effects as evidenced by down regulation of Wnt signaling target genes concerned with antiapoptosis, mitogenesis, cell proliferation and cell cycle regulation, with subsequent amelioration of liver histopathological picture.

Our results also agreed with those reported by Qiao *et al*. who stated that there is inhibitory effect of hMSCs on HepG2 hepatoma cells and human liver cancer cell lines (H7402cells) through down regulation of PCNA, survivin and β-catenin genes in both liver cancer cell lines when treated with mesenchymal stem cell line (Z3) conditioned medium.[21]

The present work investigated the effect of the co-culture medium on HepG2 cells. This was done to see if co-culture of HepG2 with MSCs would encourage the MSCs to produce factors different from those produced from MSCs alone (MSCs conditioned media). This differs from the work done by Qiao *et al*.[21] who studied the direct effect of MSCs when co-cultured with malignant cells on the latter. Our interest in this work is to investigate the effect of the conditioned media, whether of MSCs alone or if co-cultured with HepG2 cells.

Despite their distinct origins, stem cells and tumor cells share many characteristics.[23,24] In particular, they have similar signaling pathways that regulate self-renewal and differentiation, including the Wnt, Notch, Shh and BMP pathways that determine the diverse developmental fates of cells.[25,26] The Wnt signaling pathway has an important role in stem cell self-renewal and differentiation. Aberrant activation of the Wnt signaling pathway has been implicated in human tumor progression.[27,28] The PCNA, survivin and β-Catenin genes are all targets of Wnt signaling.[29,30]

Zhu *et al*.[31] demonstrated that MSCs have an inhibitory effect on tumor proliferation by identifying that DKK-1 (dickkopf-1) which was secreted by MSCs, acts as a negative regulator of Wnt signaling pathway and is one of the molecules responsible for the inhibitory effect of MSCs on tumor. MSCs also up regulate the mRNA expression of cell-cycle negative regulator p21 and apoptosis-associated protease caspase-3, resulting in a G0/G1 phase arrest and apoptotic cell death of tumor cells.[32]

Similar results were obtained by Lu *et al*.[33] who showed that MSCs exhibited a number-dependent growth inhibitory effect on murine tumor cell lines *in vitro* and inhibited the growth of ascitogenous hepatoma cells *in vivo* without host immunosuppression.

Moreover, results of the present study also showed that conditioned media and co- culture media decreased expression of telomerase gene in HePG2 cell line. Telomerase re-expression is found in 85% of human malignant tumors.[34] The expression of telomerase is important to cell proliferation, senescence, immortalization and carcinogenesis.[35] Deng and coworkers proved that both of liver telomerase activity and total RNA increased during hepatoma progression.[36]

Transforming growth factor- β 1 (TGF β) has been shown to induce down-regulation of telomerase activity.[37] TGF β down-regulated human telomerase reverse transcriptase (hTERT) expression in normal smooth muscle cells, lung fibroblasts, human keratinocytes (HaCaT), primary erythroid precursors, UT7 erythroleukemia cells, immortal kidney epithelial cells, breast cancer cells and cervical cancer cells[38] Human MSCs have been shown to display immunosuppressive properties on T-lymphocyte proliferation induced by allogeneic cells,[39] this phenomenon was reported to be mediated by the production of cytokines, in particular TGF-β1.[40] This finding could explain our findings that conditioned media from MSCs significantly down regulate telomerase gene expression. This finding could be due to secretion of TGF-β by MSCs.

As regards VEGF gene expression, our results showed that addition of MSC conditioned media and co-culture conditioned media significantly increased its expression.

It has previously been shown that co-culture of MSCs with Kaposi sarcoma (KS) cells had no effect on vascular endothelial growth factor (VEGF) promoter activity within KS cells or overall VEGF secretion by KS cells.[7] However, El-Menoufy *et al*. found that MSCs expressed VEGF in their media. The authors also demonstrated that mesenchymal stem cell transplantation may accelerate oral ulcer healing, possibly through the induction of angiogenesis by increasing VEGF expression[41]

Angiogenic support provided by MSCs is fundamental for recovery of damaged tissues. The pro-angiogenic effect of MSCs has been demonstrated in a murine model of hind limb ischemia.[42] In that study, the authors detected basic fibroblast growth factor (bFGF), VEGF, placental growth factor (PlGF), and MCP-1 in MSC-conditioned medium, and also verified the presence of bFGF and VEGF around the infused cells in situ after local administration. Recently, some populations of BM-derived MSCs have been shown to support the formation of vessel-like structures by endothelial cells in vitro in a medium devoid of the angiogenic factors VEGF-A, bFGF and IGF-1; in this system, MSCs provide, in addition to soluble angiogenic factors, extracellular matrix components that serve as a substrate for endothelial cells.[43]

Conclusions

The present study showed a possible beneficial effect of hMSCs conditioned media and co-culture conditioned media on liver malignant cells via the secretion of soluble factors that are involved in the Wnt signaling pathway with subsequent decrease in proliferation of malignant cells and decrease in expression of certain genes involved in tumorigenesis (PCNA, Survivin, β-Catenin and telomerase genes).

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