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Effect of bone marrow–derived mesenchymal stromal cells on hepatoma

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Abstract

Background aims. The aim of the study was to evaluate the effect of mesenchymal stromal cells (MSCs) on tumor cell growth *in vitro* and *in vivo* and to elucidate the apoptotic and anti-proliferative mechanisms of MSCs on a hepatocellular carcinoma (HCC) murine model. *Methods.* The growth-inhibitory effect of MSCs on the Hepa 1–6 cell line was tested by means of methyl thiazolyl diphenyl-tetrazolium assay. Eighty female mice were randomized into four groups: group 1 consisted of 20 mice that received MSCs only by intrahepatic injection; group 2 consisted of 20 HCC mice induced by inoculation of Hepa 1–6 cells into livers without MSC treatment; group 3 consisted of 20 mice that received MSCs after induction of liver cancer; group 4 consisted of 20 mice that received MSCs after induction of liver cancer on top of induced biliary cirrhosis. *Results.* MSCs exhibited a growth-inhibitory effect on Hepa 1–6 murine cell line *in vitro*. Concerning *in vivo* study, decreases of serum alanine transaminase, aspartate transaminase and albumin levels after MSC transplantation in groups 2 and 3 were found. Gene expression of α-fetoprotein was significantly downregulated after MSC injection in the HCC groups. We found that gene expression of caspase 3, P21 and P53 was significantly upregulated, whereas gene expression of *Bcl-2* and survivin was downregulated in the HCC groups after MSC injection. Liver specimens of the HCC groups confirmed the presence of dysplasia. The histopathological picture was improved after administration of MSCs to groups 2 and 3. *Conclusions.* MSCs upregulated genes that help apoptosis and downregulated genes that reduce apoptosis. Therefore, MSCs could inhibit cell division of HCC and potentiate their death.

Key Words: *apoptosis, gene expression, hepatocellular carcinoma, MSCs*

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignant disease worldwide and the third greatest cause of cancer-related death (1). The etiology of HCC has been related to a variety of diseases such as viral hepatitis (2), alcoholic hepatitis (3), nonalcoholic fatty liver disease (4) and metabolic syndrome, including diabetes mellitus (5). HCC is strongly associated with chronic hepatitis and cirrhosis (6). Most cases of HCC, approximately 80%, occur in combination with underlying cirrhosis (7); less than 10% are observed in non-cirrhotic livers, rarely without hepatitis (8). Notably, once cirrhosis is established, there is no proven effective HCC prevention (9). Malignant hepatocellular transformation is characterized by a shortened half-life and increased proliferation and regeneration of hepatocytes secondary to ongoing inflammation (10). This leads to accumulation of genomic mutations and instability, alterations that sometimes accumulate in a neoplastic phenotype (11).

Dysregulation of the balance between proliferation and cell death represents a pro-tumorigenic principle in human hepatocarcinogenesis. Molecular alterations were reported for HCC that induce an imbalance in the regulation of apoptosis. Alterations in the expression and/or activation of p53 are frequent in HCC cells, which confer on them

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resistance to chemotherapeutic drugs. Although the expression of some pro-apoptotic genes is decreased, the balance between death and survival is dysregulated in HCC mainly because of over-activation of anti-apoptotic pathways (12). Indeed, some molecules involved in counteracting apoptosis, such as *Bcl-2* and survivin, are over-expressed in HCC cells. It was indicated that inflammatory processes, as well as the epithelial-mesenchymal transitions that occur in HCC cells to facilitate their dissemination, are related to cell survival. Therefore, therapeutic strategies to selectively inhibit anti-apoptotic signals in liver tumor cells have the potential to provide powerful tools to treat HCC (12).

Mesenchymal stromal cells (MSCs) have been identified as bone marrow–derived cell populations that can differentiate into mesodermal cell lineages that are easily isolated and propagated *in vitro*. They can differentiate into a number of mesodermal cell lineages including bone, cartilage, stroma, adipose, connective tissue, muscle and tendon. Therefore, MSCs that maintain their capacity for self-renewal contribute to a wide variety of endogenous organ and tissue repair (13). Despite their distinct origins, stem cells and tumor cells share many characteristics (14). In particular, they have similar signaling pathways that regulate self-renewal and differentiation, including the Wnt, Notch, sonic hedgehog and bone morphogenetic proteins pathways (15). Wnt signaling regulates genes that are involved in cell metabolism, proliferation, cell-cycle regulation and apoptosis (16). Survivin and *Bcl-2* genes are targets of Wnt signaling (17).

Regarding the mechanism of interaction between MSCs and tumor cells, Khakoo *et al.* showed that human MSCs can inhibit proliferation of some tumor cells *in vitro* through activation of the Akt protein kinase within some but not all tumor and primary cell lines (18). Furthermore, it was reported that MSCs produced transient arrest of tumor cells in the G1 phase of the cell cycle accompanied by a reduction in the apoptotic rate (19).

The present work aimed at evaluating the effects of MSCs on tumor cell proliferation *in vitro* and the *in vivo* progression of liver cancer and to investigate the mechanistic actions of MSCs in tumor suppression by assessing the gene expression profile of caspase-3, P21, P53, survivin and *Bcl-2*.

Methods

This study was performed in the stem cell research laboratory in the Medical Biochemistry and Molecular Biology Department, in collaboration with Histology and Cell Biology, Pathology and Internal Medicine Departments, Faculty of Medicine, Zagazig University, Zagazig, Egypt.

Preparation of bone marrow–derived MSCs

Mouse stromal cells were isolated according to a protocol modified from Pulavendran *et al.* (20), with some modifications. Bone marrow cells were collected by flushing the femurs and tibias of 6-week-old BALB/c male mice with the use of culture medium [low-glucose Dulbecco's modified Eagle's medium (DMEM, 1.0 g/L glucose; Lonza Bioproducts, Basel, Switzerland) supplemented with 10% fetal bovine serum (Lonza Bioproducts)]. After aspiration, a 29-gauge needle was used to disturb aggregation; the whole aspirate was then centrifuged at 2000 rpm for 10 min. The pellet was seeded in 25-cm^2 culture plate with the culture medium, supplemented with 1% penicillin streptomycin–Amphotericin B Mixture (10 IU/10 IU/25 µg, 100 mL; Lonza Bioproducts). Cells were cultured at a concentration of 5000/cm^2/0.2–0.3 mL of media and then were incubated at 37°C in 5% humidified Co_2 in a Co_2 incubator (Heraeus, Langenselbold, Germany). Non-adherent cells were eliminated by half medium change at 1, 2 and 3 days, and the whole medium was replaced with fresh medium every week. The cells were grown for 2–3 weeks until 80–90% confluence. The whole adherent cells were detached by trypsinization with 0.25% trypsin/ethylene diamine tetra acetic acid (EDTA) (trypsin, 1:250; EDTA, 1 mmol/L; Lonza Bioproducts) and then were re-plated. Third-passage cells were used for experiments (Figure 1). Cell cultures were routinely assessed with the use of an inverted microscope, and cell viability was determined by means of trypan blue staining.

MSCs in culture were characterized by their adhesiveness and fusiform shape, which was detected by means of an inverted microscope and through determination of surface markers of bone marrow MSCs. The latter was performed through evaluation of the positive expression of non-hematopoietic origin [by use of the monoclonal antibodies that recognize an epitope on endoglin (CD105, CD44)] and the negative expression of hematopoietic markers, such as CD34, which were analyzed by means of flow cytometer according to Calabro *et al.* (21).

Preparation of mice (Hepa 1–6) cell line

Hepa 1–6 cells were obtained from American Type Culture Collection and were grown in a sterile 50-cm^2 tissue culture flask in complete medium containing DMEM supplemented with 10% fetal bovine serum 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.