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Correlation of micro vessel density and c-*Myc* expression in breast tumor of mice following mesenchymal stem cell therapy

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ABSTRACT

Stem cell therapy for degenerative diseases has been established; however there are controversies over the treatment of solid tumors with stem cell transplantation. In the present study, the anti-tumor action of mesenchymal stem cells (MSCs) has been examined in a mouse model of breast cancer with emphasize on tumor growth, angiogenesis and c-*Myc* expression in breast tumors. For this purpose, MSCs were isolated from bone marrow of Balb/c mice and characterized. A Balb/c mouse model of breast cancer was developed and subjected to cell therapy intra venous (I.V) or intra tumor (I.T) with MSCs. Tumor growth was measured using a digital caliber for until the end of experiment (30 days). Then the mice were sacrificed and their tumors were removed and processed for histopathological examination, immunohistochemical assay of CD31 and measuring of c-*Myc* expression using quantitative PCR. Detection of the labeled-MSCs in tumors following injection of the cells (I.V or I.T) clearly showed the homing of MSCs into tumors. Tumor growth in case of MSC-treated mice by I.V and I.T routes was inhibited by approximately 28% and 34% respectively compared to controls. The suppression of angiogenesis was reflected in Micro Vessel Density (MVD) following I.V or I.T delivery of the MSCs. c-*Myc* gene expression in tumor tissues of mice treated I.V or IT with MSCs was down-regulated to 28.0% and 16.0% respectively compare to control groups. In conclusion, growth inhibition of breast tumors in mice due to MSC therapy is associated with modulation of c-*Myc* activation and angiogenesis markers.

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1. Introduction

There is an increasing trend in incidence and mortality of breast cancer in women worldwide (Youlden et al., 2012). Most of the breast cancers (95%) are carcinomas that arise from breast epithelial cells. Breast cancers mainly occur in ductal or lobular epithelium which appear as in situ carcinomas or invasive (or infiltrating) carcinomas (Richie and Swanson, 2003). Both the epithelial cells and resident stem cells in breast tissue are main targets for genetic and epigenetic changes. Proliferation and invasion of breast tumor cell associated to activation of oncogenes and suppression of tumor suppressor genes as a result of which the cell cycle and cell progression are disturbed (Bombonati and Sgroi, 2011).

Surgery, chemotherapy, radiotherapy and targeted therapy are conventional methods that currently used for breast cancer treatment. Currently based on the tumor phenotype, breast cancer is treated. For instance, modulators of estrogen receptor (ER) and aromatase inhibitors such as tamoxifen and anastrozole are currently used in pre-clinical and clinical treatments of ER-positive breast cancer. Also trastuzumab and lapatinib are prescribed in the treatment of HER2-positive breast cancers for human (den Hollander et al., 2013; Goldhirsch et al., 2011). Pathways that promote tumor growth and invasion of carcinoma cells and pathways related to cell death and abnormal angiogenesis are major targets for breast cancer treatments (Schlotter et al., 2008).

In recently years it has been demonstrated that stem cell therapy can efficiently reduce the tumor growth in different animal models. In these studies animal models of cancer have been treated with mesenchymal stem cells (MSCs) to find out the therapeutic effects of MSCs. In 2006, Khakoo et al. used Kaposi's sarcoma mouse model for I.V treatment of MSC. A significant regression in tumor volume was observed which was assigned to the inhibitory effects of MSCs on Akt protein kinase activation within the tumor cells (Khakoo et al., 2006).

Also the effect of stem cell therapy (human skin-derived stem cells) in a mouse model of glioblastoma showed that as a result of stem cell therapy there was a significant inhibition in tumor growth, reduction of tumor vessel density and decrease in angiogenic sprouts (Pisati et al., 2007). Likewise Otsu et al. showed that

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MSCs injected into mouse model of melanoma caused a significant reduction in tumor-associated angiogenesis and tumor growth (Otsu et al., 2009).

The efficiency of MSC therapy in controlling breast cancer growth and metastasis in a mouse model has also reported by Sun et al. The MSCs were isolated from umbilical cord blood (UCB) as well as adipose tissue (Sun et al., 2009).

Most recently we have compared the efficiency of endothelial cells differentiated from MSCs as well as their progenitor MSCs in controlling breast tumor in a mouse model. The results showed that both MSCs and endothelial cells derived from them are capable of inhibition tumor growth. Stem cell therapy of breast tumors was found to be associated with decrease in expression of angiogenesis marker i.e., vascular endothelial growth factor receptor (VEGFR) (Adelipour et al., 2016).

Cancer cells and the surrounding microenvironment such as tumor angiogenesis and immune cells as factors and signals released by tumor cells have been considered as potential targets of cancer treatment. However, very little is known about the impact of stem cell therapy of malignant tumors on the expression of these genes and their downstream pathways.

C-*Myc* is believed to play a key role in cellular proliferation, transformation, or apoptosis and cancer related signal transduction. The changes in expression of c-*Myc* as an oncogene has been investigated in different malignancies (Dang, 1999). The role of c-*Myc* in tumorigenesis of embryonic stem cells (ES) was further confirmed using c-*Myc* knock out ES cells in tumor progression (teratoma) and vascularization (Baudino et al., 2002).

Experimental evidences show that c-*Myc* expression can act as a regulating factor in angiogenesis and modulation of VEGF expression. The role of *c-Myc* in vascularization and angiogenesis during tumor development has also been reported (Baudino et al., 2002; Knies-Bamforth et al., 2004).

In spite of the studies on the stem cell therapy on breast tumor growth in animal models there are two important issues to be addressed which can help future studies. In the present study, the mode of action and efficiency of MSC therapy by two routes of administration (I.V and I.T) in controlling mouse breast cancer has been studied. Moreover, for the first time in this study the impact of MSC therapy, tumor-associated vascularization and c-*Myc* activation in breast tumors has been investigated.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's- Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were from Gibco, USA. Penicillin Streptomycin (Pen Strep) and trypsin-EDTA were from Bio-idea, Iran. 4′,6-diamidino-2-phenylindole (DAPI) and 3,3′-Diaminobenzidine (DAB) were from Sigma, USA. Anti-CD31 antibody: (sc-1506) was from Santa Cruz, USA. Polyclonal Goat Anti-Rabbit Immunoglobulins/HRP: (P 0448) was from Dako, USA. Anti-GFP antibody: (Cat.ab1218) was the products of Abcam, UK. RNA extraction kit (Gene All Hybrid-R) and cDNA synthesis kit (HyperScript RT premix) were the products of Gene All, South Korea. PCR master mix was the product of Ampliqon, Denmark.

2.2. Preparation and characterization of MSCs used for stem cell therapy

MSCs were routinely isolated from the mouse (Balb/c) bone marrow as described previously. The cells were cultured in DMEM supplemented with 10% FBS and 1% streptomycin at 37 °C in CO2 incubator (5% CO2). The cells in their third passage with 80% confluency characterized by identification of their surface markers as well as their differentiation potential into adipogenic and osteogenic lineages. Expression of selected markers on MSCs namely; CD29, CD105 and CD45 were determined by flow cytometry (Applied Biocystems, USA). The MSCs were also checked for their differentiation potential into adipocytes and osteocytes according defined protocol as described in our previous publication (Kazemnejad et al., 2009).

2.3. Induction of mice model of breast cancer

In this study initially four female Balb/c mice were injected with 4T1 cell line (Cell Bank, Pasteur Institute of Iran) to induce breast cancer in their left flank region. The protocol is used in this study is as described in our recent publication (Adelipour et al., 2016). Briefly; tumors developed in mice were removed after 2 weeks and used for implantation to other mice which were considered as positive control and treated groups in the current study. For this purpose inbred female Balb/c 6 weeks old mice weighing 15–17 g were used to induce isogenic experimental model of breast cancer. Altogether 40 mice divided into four groups (n = 10), they were slightly anesthetized before receiving tumor graft (1–2 mm) following the procedure described previously. The first sign of tumor formation was noticed in mice 2–3 days after the grafting. Stem cell therapy was started when the size of tumors reached <7 mm (usually achieved one week after tumor grafting). The stem cell-treated groups received MSCs (1.5×10^6 cells in 100 μl of PBS/mice) either via I.T or I.V injections. Two groups of mice (n = 10) were considered as control groups received only PBS via I.V or I.T route. This study was approved by the Medical Ethics Committee of Tarbiat Modares University. The mice were monitored daily by estimation of tumor size using a digital caliper. The animals were monitored routinely and continued for 30 days. Thereafter the animals were sacrificed and tumors were removed and processed for histopathological and molecular assays. Small piece of the tumor tissues was fixed in aqueous formaldehyde (10%) and stained with H&E. The sections were examined by pathologist and focal necrosis for 10 selected fields was identified by pathologist under light microscope (magnification x400). The following scoring numbers were given to each sample: 0 (no necrosis), +1 (<10%), +2 (10%-40%) and +3 (>40%). The sections were also investigated for vascular invasion as an index of tumor cells present in the blood vessels.

2.4. Immunohistochemistry (IHC) for GFP-labeled MSCs

MSCs (50–70% confluent) were transduced with lentivirus carrying GFP gene in presence of 8 μg/mL polybrene for 24 h in order to increase the efficiency of transduction. The MSCs transduced with the lentiviral vector were checked for the presence of cells with GFP signals under fluorescent microscope. Then, the cells expressing GFP were selected from a selective medium containing puromycin (1.5 mg/mL) for 14 days (Kenarkoohi et al., 2014). Wherever indicated each mouse was injected (I.V or I.T) with approximately 1.5×10^6 cells in 100 μl of PBS.

After one week, animals were sacrificed by cervical dislocation and tumors were surgically removed and processed for fixation in paraffin blocks. Serial sections (4 μm) were prepared from each block using a microtome. The sections were then subjected to IHC using anti-GFP antibody (1:100) before observation under a fluorescent microscope. Likewise, IHC was performed on tumor biopsies prepared from control group (mice bearing tumors but untreated with stem cells).

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