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Tumor suppression effects of myoepithelial cells on mice breast cancer

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ABSTRACT

Several studies have assumed that myoepithelial cells (MECs) loss may contribute to epithelial tumor induction and/or progression. We adopted an in vitro assay and a syngeneic mice breast cancer model with histological and molecular characteristics resembling human lesions to evaluate tumor suppression effects of MECs. Flow cytometric, cell viability, blood chemistry, transmission electron microscope, immunohistochemistry and qRT-PCR assays were performed at the end of the study. We demonstrated that MECs could significantly suppress the viability of cancer cells at different time points ($P<0.05$). At the end of the fourth and fifth weeks, treated mice had smaller tumor volume compared with control animals. Average tumor volume was significantly less in treated groups than control group at days 21 (0.38 ± 0.19 vs. 1.99 ± 0.13 cm^3), 28 (0.57 ± 0.3 vs. 2.5 ± 0.37 cm^3) and 35 (0.7 ± 0.35 vs. 2.65 ± 0.4 cm^3) after tumor cell injection ($P<0.05$). No hematological, hepatocellular, and renal toxicities were seen in MECs treated groups. Ultrastructural features revealed severe relationship between adjacent tumoral cells and loose interconnections of neoplastic cells in treated group. Immunohistochemical examinations of breast tumors showed high p63 and low alpha-smooth muscle actin protein expression in treated mice compared to control ($P<0.05$). MRNA expressions of TNF-α, smooth muscle–myosin heavy chain, connexin 43, and maspin were significantly up-regulated in breast tumor tissues in treated group compared to control ($P<0.05$). VEGF and alpha-smooth muscle actin mRNA expression were reduced in treated animals ($P<0.05$). The present study highlighted the potential tumor suppression effects of MECs on breast cancer in a typical animal model.

1. Introduction

Cell therapy facilitates therapeutic modalities at various clinical clues in multiple organs based on some main principles. Several cell lines have the capacity to release soluble factors such as cytokines and growth factors to improve organ self-healing. Surprisingly, the importance of myoepithelial cells (MECs) in breast cancer has always been underestimated. These cells appear to have dual functions as tumor suppressor and promoter. MECs are a specialized combination of muscular and epithelial cells capable of contracting mammary ducts to push milk and a system of secretory apparatus of the mammary gland (Gage, 1998; Pandey et al., 2010). They considerably contribute to basement membrane creation, moreover, their myogenic differentiation is responsible for contractile phenotype mediated by various substances such as oxytocin (Alizadeh and Mirzabeglo, 2013; Imanieh et al., 2014; Murrell, 1995). Several studies have assumed that intact MECs are essential determinant of normal breast differentiation, and loss of their functions may contribute to induction and/or progression of epithelial tumors (Pechoux et al., 1999; Slade et al., 1999). While epithelial cells are susceptible targets for transforming events leading to cancer, MECs are still resistant. Although evidence proposes MECs suppressive effects on tumor growth, invasion and angiogenesis, their role remains a main puzzle in breast cancer biology (Alizadeh et al., 2014). Indeed, a number of myoepithelial-specific proteins called tumor-suppressive proteins such as alpha-smooth muscle actin (SMA), smooth muscle myosin heavy chain (SM-MHC) (Okamoto-Inoue et al., 1999), calponin, caveolin-1 (Lee et al., 1998), connexin 43 (Hirschi et al., 1996), maspin (Zou et al., 1994), and activin (Liu et al., 1996) have shown to inhibit epithelial tumor formation. Therefore, MECs may have an important role in paracrine regulation of normal and tumor cells by influencing the

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epithelial and luminal compartments, and ultimately altering breast tissue microenvironment.

To better characterize the MECs role in tumor progression, we adopted an in vitro and a syngeneic mice breast cancer model with histological and molecular characteristics resembling human lesions in a typical animal model. In fact, investigators have revealed that the myoepithelium in mice can be targeted with special molecules using mammary tumor models. Development of new therapies of human breast cancer requires suitable animal models. Orthotopic models of cell therapy have additional advantages including retention of differentiated structures within the tumor, vascular growth differences, realistic tissue pharmacokinetics at the tumor site and metastatic spread (McConville et al., 2007). Further studies to understand the exact molecular mechanisms of MECs suppressive tumor function may lead to attain a novel therapeutic target for breast cancer.

2. Materials and methods

2.1. Materials

Mouse mammary adenocarcinoma cell line (MC4-L2) was a gift from Buenos Aires University, Argentina (Lanari et al., 2001). Methylthiazol tetrazolium (MTT), phosphate-buffered saline (PBS) solution, Ketamine and Xylazine were purchased from Sigma Aldrich Co. (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were from Invitrogen.

2.2. Mouse MECs isolation

Inbred female BALB/c mice (7–9 weeks old) were placed on their back on a corkboard after euthanasia, then pinned in place through the feet, and swabbed with 70% ethanol. A ventral midline incision through the skin was made to expose mammary glands (Rasmussen et al., 2000). The mammary glands were dissected from the skin using a scalpel, starting from the proximal area close to the nipple till to the distal end of the gland, and carefully separated from the peritoneum with a blunt-edged instrument. Dissected tissues were kept in tissue dishes filled with PBS and diluted antibiotics floating on ice cubes. Immediately after delivery, sections of the mammary tissues were transported to the laboratory in sterile Hank's balanced salt solution contained 40 μg/ml of gentamicin at room temperature. The pieces were then rinsed several times with sterile phosphate buffered saline containing 2 μg/ml amphotericin B, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.3. Mammary gland digestion and MECs recovery

Mammary gland pieces were transferred to a 10-cm sterile petri dish. Tissues were mechanically minced with two scalpels inside the petri dish under sterile conditions. They were then transferred to a small flask containing PBS (10 ml/g tissue) and antibiotics. Appropriate volume of 1% collagenase was added, and the tissues were stirred at 37 °C for 30–90 min. Tissue digestion was checked starting at 30 min by aseptically removing small aliquots under the low-power microscopic examination. The desired endpoint was an epithelial preparation with no visible tissue pieces and more than 80% of epithelial organoids free of adhering stromal tissue. Then, 20 ml of F12 plus 5% FBS were added to samples digest, and allowed the clumps to be settled for 2 min. The supernatant centrifuged at 1500*g* for 5 min to prepare mammary epithelial cells and washed three times with F12 plus 5% FBS. Cells were kept in a humidified incubator setting at 37 °C in a medium refreshing three times a week. MECs were meticulously separated after organoids administration in primary culture.

2.4. Cell labeling

To track proliferation, MECs were labeled with vital dye CellTracker CM-DiI, according to the manufacturer's protocol. To test whether fluorescent nuclear track detector interferes with standard fixation and staining procedures, hybrid detector cell layer was labeled with a series of dyes. Cells were labeled with a fluorescent dye, Cell-tracker CM-DiI Molecular Probe (Cat. No. C700) at a concentration of 1.5 μg in Dulbecco's phosphate buffered saline (DPBS, 1 ml) for 8 min at humidified atmosphere first, and then for additional 15 min at room temperature. After labeling, cells gently washed twice with DPBS.

2.5. Flow cytometric analysis

Cells were incubated at 10^6/ml in L15/10% FCS with anti-CD10-fluorescein isothiocyanate (clone M1/69, BD Biosciences, Oxford, UK, 0.5 μg/ml) for 45 min at 4 °C, washed in L15/10% FCS and resuspended in L15/10% FCS/0.01% 4′, 6-diamidino- 2 phenylindole dihydrochloride (DAPI). Analysis was carried out on a BD FACSVantageSE DiVa (BD Biosciences) equipped with two coherent 90C-4 argon ion lasers (Coherent, Santa Clara, CA, USA) set at 488 nm and 333.6–333.8 nm. Samples were gated on the basis of forward-and side-scatter. Doublets and high order clumps were excluded using a time-of-flight approach, where forward-scatter-height was plotted against forward-scatter area. Routine examination of sorted cells revealed $>99\%$ single cellularity.

2.6. CO-culture of MC4-L2 and MECs to assay cell viability

To evaluate MECs effects on MC4-L2, co-cultures of MC4-L2 and MECS were performed using 6-well Transwell plates. To prevent direct cell–cell contact, MECs were seeded at a total of 5×10^5 cells on a 0.4 μm pore size Transwell filter (SPL Life Sciences Co., Ltd.). MC4-L2 was also seeded at a total of 6×10^5 cells in 6-well flat-bottom tissue culture plate. Cells were cultured in Gibco® high glucose Dulbecco's Modified Eagle Medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). All cells were grown at 37 °C in a humidified atmosphere of 5% carbon dioxide. In the first step, cells were seeded in 6 wells and allowed to be attached overnight. Seeded MECs were then transferred to the top of six wells containing MC4-L2 except one as the control and incubated. MTT assay was done to determine cell viability of triplicate wells at days 1, 3 and 5, and represented as the viability percentage using an enzyme-linked immunosorbent assay method at 570 nm.

2.7. Animals

Female inbred BALB/c mice aged 6–8 weeks old, purchased from Iran Pasteur Institute and maintained in large group houses under 12-h dark and light cycles with free access to food and water. Animals were handled according to relevant national and international guidelines of the Weatherall report, and Institutional Animal Care and Use Committee (IACUC) of Tehran University of Medical Sciences.

2.8. Tumorigenicity

MC4-L2 was trypsinized and re-suspended in 10-fold excess culture medium. After centrifugation, the cells were re-suspended in a serum-free medium. Prepared cells (1×10^6/0.1 ml) were