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Naïve human umbilical cord matrix derived stem cells significantly attenuate growth of human breast cancer cells *in vitro* and *in vivo*

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Summary

The effect of un-engineered (naïve) human umbilical cord matrix stem cells (hUCMSC) on the metastatic growth of MDA 231 xenografts in SCID mouse lung was examined. Three weekly IV injections of 5×10^5 hUCMSC significantly attenuated MDA 231 tumor growth as compared to the saline-injected control. IV injected hUCMSC were detected only within tumors or in close proximity to the tumors. This *in vivo* result was corroborated by multiple *in vitro* studies such as colony assay in soft agar and [^3H]-thymidine uptake. These results suggest that naïve hUCMSC may be a useful tool for cancer cytototherapy.

Keywords

human umbilical cord matrix stem cells; breast cancer; MDA 231 cells; xenografts; colony assay

1. Introduction

Stem cells can be derived from a variety of sources such as embryos (embryonic stem cells, ESCs), bone marrow (BMSCs), fetal tissues, cord blood, etc. Stem cells derived from these sources have significant problems associated with moral / ethical issues surrounding their derivation, which impede their adaptation into the clinical use [1-7]. Recently, it was found that umbilical cord matrix contains an inexhaustible, non-controversial source of stem cells [8-10]. With regard to moral/ethical issues, postnatal stem cells offer fewer concerns. In the United States, umbilical cords are routinely placed into biohazard waste after birth. The multipotent UCMSC are isolated from the mesenchyme-like cushioning material called ‘Wharton’s jelly’ found between the vessels of the umbilical cord [11]. UCMSC cells have several properties that make them of interest as a source of cells for therapeutic use. For example, ‘they 1) can be isolated in large quantity; 2) are negative for CD34 and CD45; 3)

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Conflict of Interest None of the authors has any financial or other interest with regards to the submitted manuscript that might be constructed as a conflict of interest.

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grow robustly and can be frozen/ thawed; 4) can be clonally expanded; and 5) can easily be engineered to express exogenous proteins [8-10].

Tumors are composed of tumor cells and nonmalignant benign cells. The “benign” tumor-associated tissue includes blood vessels, stromal fibroblasts, and infiltrating inflammatory cells [12]. Stromal fibroblasts offer structural support for malignant cells and influence the behavior and aggressiveness of cancers [12]. Malignant cells induce *de novo* formation of connective tissue in order to provide enough stroma to support cancer growth [13,14]. Our previous studies indicate that human UCMSC were attracted towards SDF-1 and VEGF under *in vitro* conditions [15]. We also found that hUCMSC engineered to secrete the cytokine IFN- β (UCMS-IFN- β) are capable of reducing growth of MDA 231 human breast carcinoma cells by inducing apoptosis [16]. It has also been shown that some un-engineered stem cells attenuate multiple tumor cells [17-19]. If un-engineered naïve UCMSC can also attenuate tumor growth, UCMSC are more clinically valuable since they are easy to prepare in relatively large quantities and are poorly immunogenic in allogeneic transplantation [20]. Furthermore, un-engineered cells are likely to be safer than genetically altered cells. In the present study, we investigated the growth attenuation potential of naïve hUCMSC on MDA 231 human breast carcinoma cells using *in vitro* cell culture studies and an *in vivo* mouse xenograft study. We have also studied the expression level of mitogen-activated protein kinases (MAPK, ERK1/2), phosphatidylinositol 3-kinase (PI3K/Akt) and apoptosis-related signaling components in MDA 231 cells alone and MDA 231 cells co-cultured with hUCMSC. Here we report that un-engineered naïve hUCMSC are capable of attenuating human breast cancer cells through attenuating primarily the Akt and MAPK pathways and stimulating the intrinsic apoptosis pathway.

2. Materials and Methods

2.1. Antibodies

Antibodies against ERK1/2, phospho ERK-1/2, p38, phosphor p38, Akt, phosphor Akt and PARP were purchased from Cell Signaling Technology (Beverly, MA). An antibody against GAPDH was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-rabbit IgG antibody was from Amersham Biosciences and ECL (Rockford, IL). Western Blotting substrate was from Pierce (Rockford, IL). Anti-human mitochondrial antibody was from Chemicon (Temecula, CA) and Alexa Fluor 488 conjugated secondary antibody from Molecular Probes (Carlsbad, CA).

2.2. Culture of human umbilical cord matrix stem cells

Human UCMSC were harvested from term deliveries at the time of birth with the mother's consent. The methods to isolate and culture hUCMSC were previously described [8]. Human UCMSC were maintained in low serum defined medium (DM), a mixture of 56% low glucose DMEM (Invitrogen), 37% MCB201 (Sigma; St. Louis, MO) and 2% fetal bovine serum (FBS, Atlanta Biologicals Inc, Georgia) containing 1x insulin transferring selenium-X (ITS-X, Invitrogen, CA), 1x ALBUMax1 (Invitrogen, CA), 1x Pen/Strep (Invitrogen, CA), 10nM dexamethasone (Sigma, MO), 100 μ M ascorbic acid 2-phosphate (Sigma, MO), 10ng/ml epidermal growth factor (R&D systems, Minneapolis) and 10ng/ml platelet derived growth factor-BB (R&D systems, MN). Cells were incubated at 37°C in an incubator with 5% CO₂ at saturating humidity. When cells reached 70%–80% confluency the cells were detached with 0.25% trypsin-EDTA (Invitrogen), the trypsin was inactivated with fresh media, and the cells were centrifuged at 250g for 5 minutes and replated at a ratio of 1:3.

2.3. Culture of MDA 231 cells

MDA 231 human breast carcinoma cells that metastasize to the lung in nude mice were obtained from M.D. Anderson Cancer Center (Houston, TX) [21]. They were maintained in DMEM (Invitrogen, CA), 1x Pen/Strep (Invitrogen, CA), and 10% FBS (Atlanta Biologicals Inc, GA), at 37°C in a humidified atmosphere containing 5% CO₂.

2.4. Xenograft study of in vivo effect of human UCMSC cells on MDA 231 tumors

To evaluate the *in vivo* effects of human UCMSC against MDA 231 human breast carcinoma cells, female CB-17 SCID mice (Harlan Laboratories, Indiana), 6–8 weeks of age were used. Mice were allowed to acclimate for 1 week after arrival. The animals were transplanted with 2×10^6 MDA 231 cells through the lateral tail vein using sterile conditions. Eight days after tumor transplantation, mice were randomized into two groups: 1) mice with MDA 231 followed by three weekly injections of saline (positive control) and 2) mice with MDA 231 followed by three weekly injections of SP-DiI labeled hUCMSC (0.5×10^6 cells per injection). A third group of mice that received saline injection alone served as a negative control. The mice were observed for signs of morbidity / death during the experiment period. All the mice were sacrificed 30 days after tumor inoculation by CO₂ inhalation and cervical dislocation. Lung weights of control and tumor-bearing animals were measured to estimate tumor burden, and lungs were sectioned (6µm) for further immunohistochemical analysis. All the animal experiments were done under strict adherence with the Institutional Animal Care and Use Committee protocol as set by Kansas State University

2.5. Immunohistochemistry staining

For immunofluorescence staining, lung tissue sections were washed with phosphate buffered saline-0.2% Triton X-100 (PBS-TX) and fixed with 70% ethanol and acetone (1:1). This was followed by washing with three changes of PBS-TX. Tissue sections were blocked with 5% normal goat serum in PBS-TX for 30 minutes, and followed by incubation with the primary antibody, anti-human mitochondrial antibody (1:1000), in PBS-TX overnight. The tissues were then washed three times with PBS-TX and incubated with Alexa Fluor 488 conjugated secondary antibody (1:1000) for 3 hours. The tissues were incubated for 30 min in Hoechst 33342 (1:100, Sigma) as a nuclear counter stain followed by a triple rinse with PBS-TX. The antigens were localized using epifluorescence microscopy (Nikon Eclipse). Images were captured using a Roper Cool Snap ES camera and Metamorph 7 software.

2.6. Anchorage-independent colony formation assay

Human UCMSC were plated in defined medium supplemented with 5% fetal bovine serum at a density of 5×10^3 cells / well in six-well tissue culture dishes. A two-layer agar system was used, in which the final concentrations of Sea Plaque Agarose (Cambrex Bio Science Rockland, Inc. Rockland, ME) were 0.9% and 0.5% in the bottom and top layers, respectively, after overnight incubation at 37°C, 5% CO₂. The top layers included 2×10^4 MDA 231 cells / well. The number of colonies with diameters over 50 µm was counted on day 10 using an automated counter equipped with an inverted microscope at 10x magnification (Olympus CKX41, Center Valley, PA).

2.7. Analysis of DNA synthesis

Cell proliferation was examined by measuring DNA synthesis using tritiated thymidine (³H-thymidine) uptake. Different numbers of hUCMSC (0.25, 1.25 and 2.5×10^4 cells) were cultured in 12-well culture plates and incubated 24 hrs for attachment. After 24 hrs, MDA 231 cells (5×10^4) were co-cultured with hUCMSC and incubated for 44 hrs. Cells were pulsed for the last 4 hrs with [³H]-thymidine (1.5 µCi/well). [³H]-thymidine

incorporation was analyzed by liquid scintillation counting using the Packard liquid scintillation counter Tri-Carb 2100TR.

2.8. Cell cycle analysis

To analyze the effect of 48 hrs hUCMSC conditioned media and hUCMSC co-culture on MDA231 cells, cell cycle analysis was carried out using propidium iodide staining. In brief, 3×10^4 HUC cells were cultured in 6-well plate and kept overnight for attachment. Next day 2.4×10^5 MDA231 cells were co-cultured with these HUCs. For condition medium experiment 2.4×10^5 MDA231 cells seeded for attachment and next day fed with 1:1(CM:DM). Cells were allowed to grow for 72 hr. At the end of incubation cells were collected and fixed in 70% pre-chilled ethanol overnight. Next day cells were collected by centrifugation and incubated in PBS containing 40 μ g/ml propidium iodide and 100 μ g/ml RNase A for 1 hr at room temperature. The fluorescence (excitation at 488 nm and emission at 585/42 nm) of 20,000 cells from each sample was measured with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA), data were analyzed using ModFit software and the results were displayed as histograms.

2.9. Western blot analysis

Total cellular protein was prepared using lysis buffer (1% TritonX-100, 0.1% SDS, 0.25M sucrose, 1mM EDTA, 30mM Tris-HCl (pH 8.0)) supplemented with protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN). Protein samples were separated by a 6-12% SDS-PAGE gel, electroblotted onto nitrocellulose membrane (Amersham Bioscience) and blocked with 5% nonfat dry milk in 0.1% Tween20 in TBS (TBST) overnight at 4°C. The membranes were washed and incubated with antibodies against - ERK1/2, phospho ERK1/2, total Akt, phospho Akt, p38, phospho p38, PARP at a 1:1000 dilution with 5% nonfat dry milk in TBST overnight at 4°C and then with a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Amersham Biosciences). The protein expression signal was detected with Pierce ECL Western Blotting substrate (Pierce, Rockford, IL). GAPDH was used as the loading control of sample by reprobing with an anti-GAPDH antibody at a 1:8000 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

2.10. Statistical analysis

Data are expressed as mean $\pm$ SE (standard error). Statistical significance was assessed by one-way ANOVA. Statistical significance was set at * $p < 0.05$; ** $p < 0.05$; *** $p < 0.001$. If not otherwise stated, all experiments reported represent three independent replications performed in triplicate.

3. Results

3.1. Human UCMS cells inhibit growth of MDA 231 tumors in SCID mice

To evaluate hUCMSC-dependent growth inhibition of human mammary tumor, MDA 231 cell xenograft study was carried out using female CB17 SCID mice. This study showed that intravenous injection of MDA 231 cells leads to the development of lung tumors and a subsequent increase in lung weight (Fig.1). However, three weekly intravenous injections of hUCMSC started 8 days after tumor transplantation significantly reduced the tumor burden as observed by measuring the lung weight (Fig 1). Immunohistochemical analysis of lungs as shown in Fig 2 depicts the specific localization of hUCMSC in MDA 231 tumor-bearing mouse lung. The red fluorescent, SP-DiI- labeled hUCMSC are located at close proximity to the anti-human mitochondrial antibody- stained MDA 231 tumor (green), but not tumor-free areas of lung or other tissues. Therefore, this xenograft study suggests that hUCMSC are

able to reach near or within the MDA 231 tumor site and attenuate the tumor burden in lungs.

3.2. hUCMSC inhibit anchorage-independent MDA 231 cells' growth

Anchorage-independent growth is a characteristic of transformed cells. In a double layer soft agar colony formation assay, colony formation of MDA 231 human breast carcinoma cells seeded on top of the bottom agar layer was significantly attenuated when naïve hUCMSC were placed under the bottom agar layer (Fig 3). This result mimics tumor growth attenuation in the mouse study and suggests that hUCMSC are capable of attenuating colony growth of MDA 231 cells through a diffusible mediator or mediators, since hUCMSC did not directly contact MDA231 cells.

3.3. hUCMSC inhibited DNA synthesis and increased G2 population in MDA 231 cells

Increase of DNA synthesis in cell is a good index for cell proliferation. Analysis of DNA synthesis, as monitored by the incorporation of tritiated thymidine, revealed that small amounts of co-cultured hUCMSC significantly and dose-dependently inhibited DNA synthesis in the MDA 231 cells (Fig 4). Furthermore, G2 populations were significantly increased in MDA 231 cells cocultured with a small number of hUCMSC or cultured with medium conditioned by hUCMSC (Fig. 5). Both the decrease in DNA synthesis and the increase of the G2 population in MDA 231 cells by either coculture with hUCMSC or with conditioned medium corroborates with inhibition of anchorage-independent colony growth and with *in vivo* tumor growth attenuation. These results suggest that hUCMSC-dependent growth attenuation of cancer cells was initiated by inhibition of the cell cycle.

3.4. hUCMSC attenuated Akt and MAPK phosphorylation in MDA 231 cells

To clarify the mechanism by which hUCMSC attenuated growth of cancer cells, Western blot analysis of multiple cell growth signaling components was carried out. Co-culture of a small number of hUCMSC with MDA 231 cells (1:30 and 1:6) significantly attenuated phosphorylation of Akt and ERK1/2 in MDA 231 cells. It was also observed that as the number of hUCMSC in co-culture increases, phosphorylations of Akt and ERK1/2 was further attenuated. Although the apoptosis index, as determined by p-38 phosphorylation and cleavage of PARP, in MDA 231 cells was slightly increased by co-culture with hUCMSC, these increases were statistically not significant. These results indicate that attenuation of the Akt and MAPK pathways is involved in the naïve hUCMSC-dependent growth attenuation of breast carcinoma cells.

4. Discussion

Increasing evidence suggests that adult stem cells can be effective therapeutic tools for various diseases including cancer [22-24]. Indeed, multiple adult stem cells engineered to express therapeutic genes are reported to be very effective in attenuating various tumors [15,21,25]. On the other hand, a few papers have reported that naïve adult stem cells have an intrinsic ability to attenuate growth of several types of cancer cells, such as Kaposi's sarcoma and glioma [17-19]. A drawback of cancer cytototherapy using engineered stem cells is an unexpected gene expression of the transfected gene, or, if viral vectors are used, mutation of the vector into a virulent form or insertion into inappropriate genomic regions. Thus, if naïve stem cells can be used for cancer cytototherapy, the safety of cytototherapy will be increased significantly.

Accordingly, the aim of the present study was to evaluate the therapeutic potential of hUCMSC. In the present study we evaluated the intrinsic therapeutic ability of hUCMSC for human malignant breast carcinoma cells *in vivo* and *in vitro*. The present study provides

strong evidence that hUCMSC can be used as a safe cancer-targeted cytotherapy for breast carcinoma.

First, we examined the attenuation effect of naïve hUCMSC on MDA 231 metastatic lung tumor growth in SCID mice. As described in the results, intravenously administered naïve hUCMSC significantly attenuated tumor growth in the lung (Fig. 1). Furthermore, immunohistochemical analysis of hUCMSC in the lung detected a large number of hUCMSC within or adjacent to tumor tissues, but not in normal areas of the lung or in other tissues. Therefore, hUCMSC appear to home to the tumor site, but not to healthy tissues. Although the homing mechanism of hUCMSC is still unclear, these data suggest that homing of hUCMSC to the tumor site should be an important step in hUCMSC-induced tumor growth attenuation. This *in vivo* mouse study suggests that naïve hUCMSC are a potentially useful tool for cancer cytotherapy. Accordingly, a potential mechanism by which hUCMSC induces cancer cell growth attenuation was examined in *in vitro* studies.

Accordingly, we have evaluated the effect of hUCMSC on the colony growth of MDA 231 carcinoma cells in soft agar. As shown in Fig.3, co-culturing hUCMSC in the bottom of the culture dish significantly attenuated colony growth of the MDA 231 carcinoma cells. Since MDA 231 cells were separated from hUCMSC by a solidified agar layer (approximately 1 mm), this growth attenuation appears to be mediated by diffusible factor(s). This hUCMSC-induced growth attenuation was further confirmed by [3H]-thymidine uptake assay in co-culture of a small number of hUCMSC with MDA231 cells (Fig. 4), suggesting that hUCMSC-induced cell growth attenuation is of relatively early onset and involves the inhibition of DNA synthesis. Indeed, analysis of the cell cycle with flow cytometry indicated that co-culture with hUCMSC or its conditioned medium increased the G2 population in MDA 231 cells (Fig.5). This thymidine uptake assay along with the cell cycle analysis strongly suggest that hUCMSC produce a mediator or mediators that attenuate cell growth, perhaps by alteration of the cell cycle of cancer cells. Although Larmonier *et al* have reported that nitric oxide plays a significant role in bone marrow mesenchymal stem cell-induced growth alteration of cancer cells [17], hUCMSC-dependent growth attenuation may not be due to nitric oxide since nitric oxide is very unstable in culture media [26]. Rather, our hUCMSC conditioned medium attenuated cell growth of MDA 231 cells and increased G2 population in MDA231 cells (Fig. 5).

Western blot analysis of phosphorylation of Akt and Erk1/2, PARP cleavage and p38 MAPK phosphorylation showed that co-culture of a small number of hUCMSC with MDA 231 cells (1:30 and 1:6) attenuated Akt and ERK1/2 phosphorylation, slightly increased caspase 3 substrate PARP cleavage, and increased p38 phosphorylation in the cell lysate. Although these signals were obtained from a mixture of cancer cells and hUCMSC, changes of these indicators appear to be primarily due to an alteration of co-cultured cancer cells since the MDA cells predominate significantly. These results suggest that a small number of hUCMSC attenuate survival of cancer cells and induce cancer cell death. It is well known that in response to a growth inhibitor, such as TGF- β , interferon- β , or TNF- α cancer cells undergo down-regulation of the cell cycle and stimulation of apoptosis. Therefore, it is conceivable that co-cultured hUCMSC produced a cancer cell cytotoxic factor or factors, thus attenuating cancer cell growth. These *in vitro* studies further suggest that naïve hUCMSC are potentially useful for cancer cytotherapy. To the best of our knowledge, the present study is the first to demonstrate that naïve hUCMSC significantly diminished viability of human mammary carcinoma cells *in vitro*.

In summary, the present study demonstrates that hUCMSC significantly attenuates growth of MDA 231 human breast carcinoma cells in culture and in a mouse xenograft study. Tumor growth attenuation appears to be associated with targeted homing of hUCMSC to

tumor tissue. The hUCMSC also attenuated DNA synthesis, increased G2 populations, and inhibited colony growth of co-cultured MDA 231 carcinoma cells. The mechanism by which hUCMSC attenuate growth of cancer cells is mainly by attenuation of Erk-1/2 and PI3K/Akt signaling and potentially an activation of intrinsic apoptosis. These results clearly indicate that naïve hUCMSC is a potential cytotherapeutic tool for breast cancer therapy.

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Fig. 1.

Effect of hUCMSC on the growth of MDA 231 human breast carcinoma xenografts in CB17 SCID mice. MDA 231 cells (2×10^6 cells) or saline were intravenously inoculated through the tail vein. Eight days after the carcinoma cell inoculation, hUCMSC (5×10^5 cells) were injected through the tail vein. Tumor burden was determined one week after the third weekly hUCMSC treatment. *, $p \leq 0.05$ as compared to the untreated MDA 231 alone group.

Fig. 2. Specific localization of hUCMSC in MDA 231 tumor-bearing mouse lung. One week after three weekly injections of SP-DiI labeled hUCMSC (5×10^5 cells) to MDA 231 tumor-bearing mice, mice were sacrificed and lungs were subjected to immunohistochemical analysis. The fluorescent micrograph shows human mitochondrial stained MDA 231 cells (green), nuclei (blue) stained by Hoechst 33342 and selective engraftment of SP-DiI labeled hUCMS cells (red) in close proximity to the MDA 231 lung tumors (green).

Fig. 3.

The colony growth of MDA 231 human breast carcinoma cells was significantly attenuated by co-culture with hUCMSC in soft agar. The hUCMSC (3×10^3) were seeded on the bottom of a 6-well culture dish one day prior to the carcinoma cell seeding. The carcinoma cells (5×10^4) suspended in 0.4% agar were seeded on top of the 0.8% bottom agar layer. Colony growth was evaluated 10 days after the carcinoma cell seeding. The panels A-1 and B-1 represent typical views of the MDA 231 colony growth in the middle layer, whereas panels A-2 and B-2 represent views of the control (no cells) and hUCMSC in the bottom of culture dish. Panel C represents a summary of the colony assay. ***, $p \leq 0.001$ as compared to the colony number of MDA231 alone group.

Fig. 4. [3H]-Thymidine-uptake into MDA 231 cells was significantly attenuated by co-culture with a small number of hUCMSC. Various numbers of the hUCMSC (0.25, 1.25 and 2.5×10^4) were seeded one day prior to the carcinoma cell (5×10^4) culture in a 12-well culture plate. The [3H] Thymidine-uptake was evaluated 44 hrs after the co-culture, as described in the Methods section. Cell numbers under the horizontal bars indicate hUCMSC co-cultured with MDA 231 cells *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ as compared to MDA231 alone.

Fig. 5.

Direct co-culture of a small number of hUCMSC and hUCMSC-conditioned medium with MDA 231 cells significantly increased G2 populations in MDA 231 cells as compared with untreated or defined medium treated MDA 231 cells. In the co-culture study, 4×10^4 hUCMSC were seeded one day prior to the 2.4×10^5 MDA 231 cells. The ratio of the two cell types was 1:6. Three days after co-culture the whole cells were lightly trypsinized and subjected to flow cytometry. Bar graphs indicate the average of two independent triplicate determinations. *, $p < 0.05$, ***, $p \leq 0.001$ as compared to the level of untreated control.

Fig. 6.

Direct co-culture of a small number of hUCMSC significantly attenuated Akt (A) and ERK-1/2 phosphorylation (B) in MDA 231 cells, whereas only slightly increased apoptosis-associated signaling of p38 MAPK (C) and PARP cleavage (D) were observed. In the co-culture study, hUCMSC were seeded one day prior to the MDA 231 cells. The ratio of two cell types was 1:30 and 1:6. One day after co-culture the whole cell lysate was prepared and subjected to Western blot analysis. In each panel, the left side picture indicates Western blot analysis, whereas the right side bar graph shows semi-quantification of expressed proteins using scanning densitometry as described in Materials and Methods. The average expression levels normalized by GAPDH level (n=3) are displayed in the histogram.