RESEARCH ARTICLE

IGF-1 from Adipose-Derived Mesenchymal Stem Cells Promotes Radioresistance of Breast Cancer Cells

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Abstract

Purpose: The aim of this study was to investigate effects of adipose-derived mesenchymal stem cells (AMSCs) on radioresistance of breast cancer cells. Materials and Methods: MTT assays were used to detect any influence of AMSC supernatants on proliferation of breast cancer cells; cell migration assays were used to determine the effect of breast cancer cells on the recruitment of AMSCs; the cell survival fraction post-irradiation was assessed by clonogenic survival assay; γ-H2AX foci number post-irradiation was determined via fluorescence microscopy; and expression of IGF-1R was detected by Western blotting. Results: AMSC supernatants promoted proliferation and radioresistance of breast cancer cells. Breast cancer cells could recruit AMSCs, especially after irradiation. IGF-1 derived from AMSCs might be responsible for the radioresistance of breast cancer cells. Conclusions: Our results suggest that AMSCs in the tumor microenvironment may affect the outcome of radiotherapy for breast cancer *in vitro*.

Keywords: Adipose-derived mesenchymal stem cells - insulin-like growth factor 1 - breast cancer cell proliferation

Asian Pac J Cancer Prev, **15 (23)**, 10115-10119

Introduction

Breast cancer is one of the most occurring cancers in women worldwide (approximately 25%) (Jemal et al., 2008). Most patients suffering from breast cancer are required mandatory radiotherapy after breast-conserving surgery or mastectomy, but the local and regional control and survival rate of breast cancer patients remains unsatisfactory (Li et al., 2013; Qu et al., 2013). Radioresistance might be the major reason for this failure in treatment.

According to previous reports, adipose-derived mesenchymal stem cells (AMSCs) isolated from human adipose tissue exhibited regenerative capabilities (Zuk et al., 2001). Owing to their ease of culture, abundant availability, and low immunity (Le Blanc and Ringden, 2007; Yang et al., 2011) AMSCs have been employed extensively in medical fields such as plastic surgery, cardiac surgery and breast surgery (Eterno et al., 2013) AMSCs have also been used for tissue damaged by radiotherapy after breast cancer surgery (Semont et al., 2006). However, AMSCs might dramatically favor breast cancer recurrence (Eterno et al., 2013). These indicated that AMSCs might not be safe for breast reconstruction or damage repair after breast cancer surgery in clinical applications.

AMSCs could promote breast cancer progression by activating HGF/c-Met signaling (Eterno et al., 2013) however, the underlying mechanism of the AMSCs on radioresistance of breast cancer cells remains unclear. AMSCs can secrete growth factors, cytokines and chemokines, such as insulin-like growth factor 1 (IGF-1). (Wang et al., 2013). One of the characteristics of breast cancers is that their abnormal signaling transduction pathways, including insulin-like growth factor 1 receptor (IGF-1R) and epidermal growth factor receptor (EGFR). signaling, which have been implicated in the radiation resistance of breast cancers in previous reports (Gee and Nicholson, 2003; Livasy et al., 2006; Taunk et al., 2010; Yerushalmi et al., 2012). The progression and metastatic capabilities of breast cancer are enhanced by the activation of both the MAPK and PI3K/AKT signaling pathways (Vincent and Feldman, 2002; Martin et al., 2012). As a mitogen, IGF-1 can activate both signaling pathways through ligand binding to receptors (tyrosine kinase insulin-like growth factor receptor). When IGF-1 is bound to the IGF-1R, the progression of breast cancer is augmented through activation of these pathways (Gooch et al., 1999; Sachdev and Yee, 2001).

In this study, we aimed to investigate the potential molecular mechanism of the crosstalk between AMSCs and breast cancer cells on radioresistance *in vitro* and to

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confirm whether AMSCs could enhance the radiation resistance of breast cancer cells.

Materials and Methods

Cell culture

AMSCs were obtained from the American Type Culture Collection (ATCC). The breast cancer cell lines MCF-7 and BT474 were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Both of the AMSCs and MCF-7 were cultured in DMEM-HG (containing 25mM glucose) 10% fetal bovine serum (FBS, Gibco, Australia) and antibiotics (100 U/ml penicillin and 100mg/ml streptomycin). The BT474 cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. All cell lines used in this study were cultured under a humidified atmosphere of 5% CO_2 at 37°C.

Cell proliferation assay

Cell proliferation was measured using the 3-(4, 5-dimethylthiazol-2-yl).-2, 5-diphenyl tetrazolium (MTT). dye reduction method as described previously (Green et al., 1984). Briefly, breast cancer cells (2×10^3). were plated into each well of 96-well plates. Twenty-four hours after incubation, culture medium or AMSCs culture supernatants were added into wells and incubated for 1-3 days. After incubation, cells were incubated with 50μl MTT solution (2mg/ml; Sigma-Aldrich, St. Louis, MO, USA) for additional 2h. Then the medium was subsequently aspirated, and the dark blue crystals were dissolved with 100μl DMSO (Sigma-Aldrich). The absorbance was measured using a microplate reader with reference wavelengths of 570 nm. Each experiment was performed with triplicate samples and at least three times independently.

Cell migration assays

Cell migration assays were performed using Boyden chambers (Corning Costar, Cambridge, MA, USA) with an 8-μm pore filter separating the top and bottom Transwell chambers. AMSCs (1×10^4 cells/200μl) were resuspended in serum-free media and added to the top chamber. MCF-7 cells (5×10^4 cells/500μl). with completely media were added to the bottom chambers. 48h after seeding, the cells that failed to migrate from the top surface of the filters were removed with cotton swabs. The cells on the bottom surface of the filters were fixed with methanol and stained with 1% crystal violet. Six random fields were counted. For treatment with irradiation, MCF-7 cells were exposed to 4 Gy irradiation and 1 h later, 5×10^4 cells/500μl were seeded to the bottom chambers as described above.

IGF-1 production in AMSC culture supernatants and breast cancer cell lines

AMSCs, MCF-7 or BT-474 cells (5×10^6) were incubated in 5-mL culture medium for 48h. The level of IGF-1 in the cell supernatants was quantitated by ELISA (Human IGF-I Quantikine ELISA Kit; R&D) according to the manufacturer's protocol. All samples were tested in triplicate independently.

Clonogenic survival assay

MCF-7 and BT474 cells were seeded onto 6-well plates at specific cell densities according to the dose irradiated. After 24h incubation, cells were pretreated with AG1024 (15μM; Calbiochem, San Diego, CA, USA). and/or IGF-1 (20ng/ml; Invitrogen, Carlsbad, CA, USA). or AMSCs supernatants followed by exposing to a range of 0-8 Gy irradiation. 24h after irradiation, AG1024, IGF-1 or AMSCs supernatants were washed away and cells were incubated for additional two weeks. Then the cells were fixed with 100% methanol and stained with 1% crystal violet. Colonies containing ≥50 cells were counted by microscopic inspection. The survival fraction was calculated as described previously (Xie et al., 2012). Survival curves were fitted to the classic multi-target single-hit model ($SF=1-(1-e^{-D/D0})^{.N}$). using GraphPad Prism software (GraphPad, La Jolla, CA).

γ-H2AX foci detection by fluorescence microscopy

Approximately 1×10^5 breast cancer cells were seeded on cover slips in 24-well plates and incubated overnight. Then cells were pretreated with AMSCs supernatants for 2h followed by exposing to a single dose of 4Gy irradiation, and incubation for additional 24h. The cells were subsequently fixed in 4% paraformaldehyde (Sigma-Aldrich) permeabilized in 0.1% Triton X-100 (Sigma-Aldrich) blocked in 2% BSA (Roche) and incubated with primary antibodies against anti-γ-H2AX (Abcam, San Fransisco, CA, USA). overnight at 4°C. The cells were then washed with PBS and incubated with a secondary antibody (goat anti-mouse-Alexa Fluor 488, Invitrogen). for 1 hour at room temperature Followed by washing with PBS and counterstaining with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). The slides were observed under fluorescence microscopy (Olympus BX51, Tokyo, Japan). For each group, the γ-H2AX foci were counted in at least 50 cells.

Antibodies and western blotting

The primary antibodies used in the current study were abbit anti-IGF-1R (1:1000; Abcam). and mouse anti-β-actin (1:1000; ProteinTech). Western blotting was performed as previously described (Liu et al., 2014).

Statistical analysis

Results represent the mean±standard deviations (SD) of at least 3 independent experiments. Student's t-test was used for comparison of the difference between two groups. One-way ANOVA was used for comparison of the difference between more than two groups. All p values lower than 0.05 were considered statistically significant.

Results

AMSCs supernatants promote proliferation of human breast cancer cells

In this study, we firstly investigated the susceptibility of breast cancer cells affected by AMSCs. By incubation with AMSCs supernatants, we observed an increased viability of MCF-7 and BT474 cells in the presence of AMSCs supernatants compared to the control group

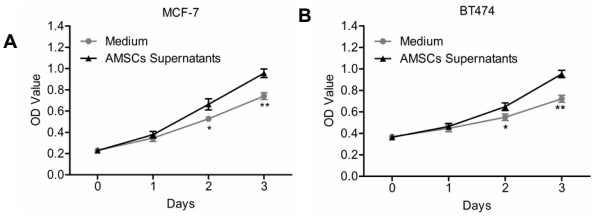

Figure 1. AMSCs Supernatants Promote Proliferation of Human Breast Cancer Cells. A) MCF-7 cells were incubated in the presence or absence of AMSCs supernatants (ratio of 1:2), and cell growth was determined 1-3 d by MTT assay. B) BT474 cells were incubated in the presence or absence of AMSCs supernatants (ratio of 1:2), and cell growth was determined 1-3 d by MTT assay. *p<0.05, **p<0.01. The bars indicate SD

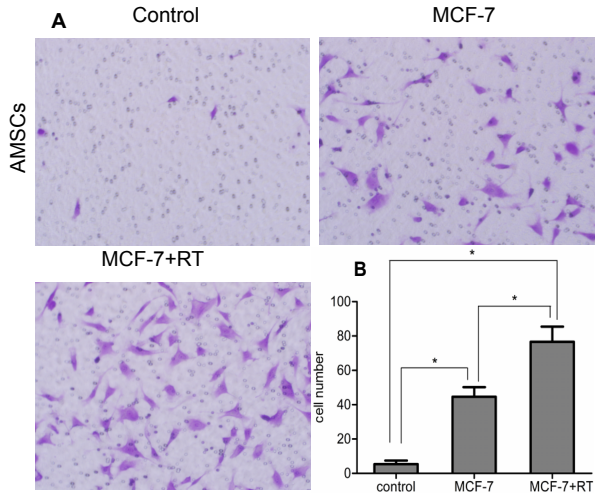

Figure 2. Human Breast Cancer Cells Induce Recruitment of AMSCs. A) Migration of AMSCs induced by MCF-7 cells. The assay was performed with triplicate samples as described above. **B)** Statistical graph of A). Data indicated mean±SD. *p<0.05

(Figure 1A and 1B). These results indicate that there seem to be some cytokines secreted by AMSCs to promote the proliferation of breast cancer cells.

Human breast cancer cells induce recruitment of AMSCs

Next, we assessed the effect of breast cancer cells on the recruitment of AMSCs. In the presence of medium alone, only a few AMSCs migrated through the filters. However, MCF-7 cells could dramatically induce migration of AMSCs, especially after irradiation (Figures 2A and 2B). These data suggest that breast cancer cells have an ability for recruitment of AMSCs, which might be associated with radioresistance of breast cancer cells.

AMSCs supernatants induce radioresistance of breast cancer cells

To confirm the effect of the supernatants of AMSCs on the radioresistance of breast cancer cells, clonogenic survival assays were performed. As shown in Figure 3A, pretreatment with supernatants of AMSCs promoted the survival fraction of both MCF-7 and BT474 cells post-irradiation.

To further evaluate the effect of the DNA damage response of AMSCs supernatants in breast cancer cells, we measured the number of γ-H2AX foci post-irradiation,

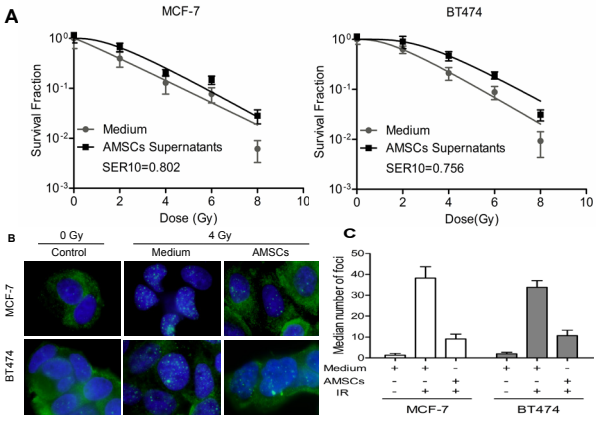

Figure 3. AMSCs Supernatants Induce Radioresistance in Breast Cancer Cells. A) The clonogenic survival assays of the MCF-7 and BT474 cells treated with AMSCs supernatants (ratio of 1:2) followed by various dose of irradiation. Data were shown as Mean±SD from three independent experiments. Curves were fitted using the classic multi-target single-hit model. SER10, sensitizer enhancement ratio at 10% survival. **B)** Radioresistance induced by AMSCs supernatants is accompanied by the lower persistence of the γ-H2AX. Immunofluorescence micrographs of the γ-H2AX foci in the presence or absence of AMSCs supernatants (ratio of 1:2) with or without irradiation. Original magnification, ×400. **C)** Statistical graph of **B)** Each bar represents the mean±SD

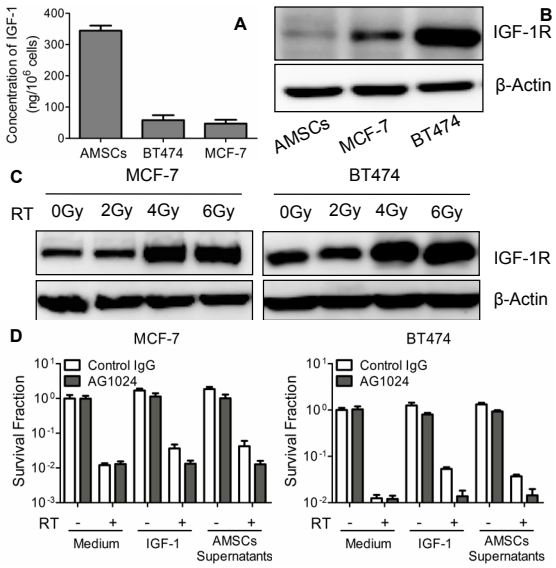

Figure 4. IGF-1 Derived from AMSCs is Correlated with Radioresistance in Human Breast Cancer Cells. A) The levels of IGF-1 secreted by MCF-7, BT474 cells and AMSCs were measured by Elisa. **B)** The expression of IGF-1R in MCF-7, BT474 cells and AMSCs was determined by Western blot. **C)** Twenty-four hours after radiation with various doses, the expression of IGF-1R in MCF-7, BT474 cells and AMSCs was detected by Western blotting. D) Survival fraction of 8 Gy irradiation of MCF-7 and BT474 cells with or without IGF-1 (20 ng/ml), AG1024 (15 μM), or culture supernatants of AMSCs (ratio of 1:2). Data are presented mean±SD

which is a well-known marker of DNA double-strand breakage and repair (Begg et al., 2011). Pretreatment with supernatants led to a dramatic reduction in the number of γ-H2AX foci post-irradiation compared to irradiation alone (Figure 3B and 3C). These results indicate that AMSCs supernatants induce radioresistance in breast

cancer cells in a manner which might be associated with the lower persistence of DNA damage.

IGF-1 derived from AMSCs is correlated with radioresistance in human breast cancer cells

Next, we examined which cytokine produced by AMSCs was responsible for the radioresistance of breast cancer cells. As shown in Figure 4A, MCF-7 and BT474 cells secreted lower levels of IGF-1 into their culture supernatants compared to AMSCs. However, the expression of IGF-1R in MCF-7 and BT474 cells were higher than that in AMSCs (Figure 4B). Twenty-four hours after various dose of radiation, IGF-1R expression in MCF-7 and BT474 cells were gradually increased (Figure 4C).

To confirm the effect of IGF-1 derived from AMSCs on the radioresistance of breast cancer cells, clonogenic survival assays were performed with or without IGF-1, anti- IGF-1R (AG1024). or culture supernatants of AMSCs. As shown in Figure 4D, after 8 Gy irradiation, the survival fraction was significantly increased in the present of the AMSCs supernatants or IGF-1 compared to irradiation alone, in MCF-7 and BT474 cells. However, this effect was reversed by treatment with anti-IGF-1R neutralizing antibody AG1024. These results revealed that IGF-1 derived from AMSCs might play a critical role in radioresistance of breast cancer cells.

Discussion

In this study, we found that AMSCs supernatants promoted proliferation and radioresistance of breast cancer cells. We also found that breast cancer cells could dramatically induce recruitment of AMSCs, especially after irradiation. Further investigation demonstrated that IGF-1 derived from AMSCs might responsible for the radioresistance of breast cancer cells. Our results suggest that AMSCs affect the outcome of radiotherapy for breast cancer.

Due to their ease of culture, abundant availability, and low immunity, the regenerative capability of AMSCs have been applied extensively in medical fields (Le Blanc and Ringden, 2007; Yang et al., 2011; Eterno et al., 2013). However, the roles of AMSCs in clinical applications after cancer surgery have been disputed intensively in the past decade (Karnoub et al., 2007; Sun et al., 2009; Klopp et al., 2010; Martin et al., 2010; Liu et al., 2011; Pearl et al., 2012; Zhao et al., 2012). More and more reports suggested that mesenchymal stem cells could promote cancer progression. Therefore, the use of AMSCs for tissue damage after cancer surgery remains hotly debated (Semont et al., 2006). As previously reported, many patients with autologous fat graft for therapy or breast reconstruction suffered cancer recurrence or progression (Perrot et al., 2010; Chaput et al., 2013; Eterno et al., 2013). The prospect of the clinical application of AMSCs for cancer therapy remains unsatisfied. In this study, we demonstrated that AMSCs supernatants could promote the proliferation and radioresistance of breast cancer cells in a manner which might be associated with the lower persistence of DNA damage.

Recent studies have demonstrated that AMSCs could favor breast cancer recurrence and progression through activating the HGF/c-Met signaling pathway (Eterno et al., 2013). However, the mechanisms underlying the relationship between AMSCs and the radioresistance of breast cancer remains unclear. IGF-1R is a transmembrane receptor tyrosine kinase involved in the development and progression of cancer whose activation strongly promotes cell growth and survival (Adams et al., 2000; Valenciano et al., 2012). IGF-1 binds to IGF-1R with high affinity (Ullrich et al., 1986). Studies indicated that IGF-1R has been associated with cell adhesion, cell motility, and tumor metastasis (Tai et al., 2003). Additionally, IGF-1R activation has also been associated with enhanced radioresistance both *in vitro* and *in vivo*, which might be due to its overexpression post-irradiation (Turner et al., 1997; Bartucci et al., 2001). Co-inhibition of EGFR and IGF-1R could sensitize breast cancer cells to radiation by inhibiting both MAPK and PI3K/AKT signaling pathways (Vincent and Feldman, 2002; Martin et al., 2012). Study also demonstrated that the circulating levels and tissue expression of IGF-I in cancer patients was higher than that in control groups (Sharma et al., 2012) indicating the activation of IGF-1 pathway in cancer patients. In this study, we revealed that there might exist a potential molecular mechanism of the crosstalk between AMSCs and breast cancer cells on radioresistance. Our data showed that the IGF-1R expression was significantly upregulated after radiation in breast cancer cells. Breast cancer cells could recruit AMSCs, which produce IGF-1 to enhance radioresistance of breast cancer cells. Furthermore, we also observed that radiation resistance induced by AMSCs-derived IGF-1 could be circumvented by an IGF-1R antibody AG1024.

In summary, our results highlight the role of AMSCs in the radioresistance of breast cancer cells *in vitro*. However, further work is required to investigate the complex mechanism of AMSCs and the tumor microenvironment in the therapeutic resistance of breast cancer.

Acknowledgements

This study was supported by National Natural Science Foundation of China Grant (31370994) and the Science and Technology Department of Guizhou Province (LKZ2012-16).

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