Human mesenchymal stem cells enhance autophagy of lung carcinoma cells against apoptosis during serum deprivation

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Abstract. Currently, some evidence suggests that human multipotential mesenchymal stems cells (hMSCs) aid tumor growth and metastasis. Nutrient deprivation and oxygen deficiency are representative characteristics of solid tumor microenvironment during the cancer development. Because the effects of hMSCs on tumors under stressful conditions have not been determined, we investigated the survival mechanisms used by stressed stromal cells on A549 and SPC-1 lung carcinoma cell lines in vitro and in vivo. An indirect culture system was used to investigate the effects of hMSCs on viability and apoptosis in starved carcinoma cells and focused on the role of autophagy in regulating the survival of carcinoma cells. The results showed that A549 and SPC-1 cells had higher viability when co-cultured with hMSCs and that this was mainly attributed to decreased apoptosis. Autophagosomes were analyzed using GFP-LC3 and electron microscopy, which showed that autophagy was significantly activated in the starved co-culture groups. However, the inhibition of autophagy by the autophagic inhibitor 3-MA significantly abrogated the apoptosis reduction in either single groups or co-culture groups under serum deprivation, which implied that the hMSCs protected against apoptosis by enhancing autophagy in lung carcinoma cells in vitro. We also observed that hMSCs promoted tumor initiation and growth in vivo. In conclusion, our study demonstrates that hMSCs can protect carcinoma cells from nutrient deprivationinduced apoptosis and promote tumor initiation and growth, and, interestingly, autophagy plays an important role in the survival of cancer cells.

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Introduction

Currently, lung cancer is the leading cause of cancer-related mortality throughout the world (1). Although there have been significant advances in cancer treatments, this malignancy remains poorly responsive to conventional therapy. Hence, it is urgent to determine the survival mechanism of carcinoma cells to develop more efficient therapies for patients.

Human mesenchymal stem cells (hMSCs) are pluripotent progenitor cells that reside within the adult bone marrow. They have self-renewal capacity, long-term viability and can differentiate into the adipocytic, chondrocytic or osteocytic lineages (2-4). Although hMSCs reside predominantly in the bone marrow, they are also distributed throughout many other tissues, where they are thought to function as local sources of dormant stem cells (5). After injury or chronic inflammation, the wounded tissue would release specific endocrine signals that are then transmitted to the bone marrow, leading to the mobilization of multi-potent hMSCs and their subsequent recruitment to the damage site (6). Moreover, recent evidence has indicated that hMSCs are recruited and incorporated within the connective tissue stroma of tumors (7-10). In the tumor microenvironment, hMSCs can secrete several tumor growth-promoting factors, favoring tumor growth, enhancing tumor vessel formation, promoting cancer metastasis and creating tumor stem cell niches (11-14). The ability of hMSCs to home to sites of injury and tumors has encouraged investigation of these cells as potential therapeutic tools.

Nutrient deprivation and oxygen deficiency are representative characteristics of the solid tumor microenvironment during cancer development (15,16). Autophagy is a wellestablished mechanism for degrading cytoplasmic proteins, macromolecules, and organelles to provide a nutrient source to promote the survival of cells that are under metabolic stress (17,18). Starvation increases the number and size of autophagosomes in many tissues, suggesting that autophagy is a critical component of the body's response to nutrient deprivation and amino acid/fuel homeostasis. Autophagy has been implicated in a number of different physiological and pathological conditions, including development, differentiation, immunity, aging and cell death (19,20). In addition, accumulating evidence demonstrates interesting links between

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autophagy and tumorigenesis, tumor progress and chemoresistance (21,22). In particular, the regulation of autophagy in carcinoma cells is complex because it can enhance tumor cell survival in response to certain stresses.

Because stromal cells play an important role in solid tumor development, there is a significant gap in our understanding of the relationship between stromal cells and carcinoma cells under stressful conditions, such as hypoxia or nutrient deprivation. In this study, we investigated the influence of hMSCs on A549 and SPC-1 cells under nutrient deprivation and the role of autophagy in this context, in lung carcinoma cells. Our study demonstrates that hMSCs can protect carcinoma cells from nutrient deprivation-induced apoptosis, and interestingly, autophagy plays an important role in this protection. Further, we found that hMSCs promoted earlier tumor initiation and growth *in vivo*. This result indicated that protection by stromal cells is a factor that helps lung carcinoma cells survive and proliferate continually in the ischemic microenvironment, even under extreme nutrient limitation.

Materials and methods

Cell culture and reagents. The human lung carcinoma cell lines A549 and SPC-1 were purchased from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (DMEM/F12) (Gibco, Invitrogen), containing 10% fetal bovine serum (FBS) (Gibco, Invitrogen), 100 U/ml penicillin and 100 μ g/ml streptomycin in a humidified incubator under 95% air and 5% CO₂ at 37°C. 3-MA and DMSO were purchased from Sigma-Aldrich. Both DMSO and 3-MA were used at 5 mM.

HMSCs were isolated from hip aspirates of two male healthy donors with locally approved informed consent. The marrow was diluted twice with phosphate buffered saline (PBS) and then isolated by Percoll (Sigma-Aldrich) density-gradient centrifugation (specific gravity 1.073). Primary cells were collected and incubated in DMEM/F12 containing 10% FBS, 0.2 mmol/l glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin under 95% air and 5% CO₂ at 37°C. After 48 h, the medium was replaced and non-adherent cells were discarded. After 3-5 passages, the cells met the minimal criteria for defining multipotent mesenchymal stromal cells with typical CD45-, CD34-, CD14-, CD19-, HLA-DR-, CD73+, CD90+ and CD105+ expression (23), as identified by flow cytometry (data not shown). Passages three to five hMSCs were used in this study.

Cell co-culture or hMSC SD-conditioned medium treatment. Co-culture systems were established using 6-well transwell (0.4 μ m pore, Corning) plates. hMSCs were plated at 1x10⁵ cells per insert and A549 or SPC-1 cell suspensions 2x10⁵ cells per well were placed in the lower compartment of the culture well. The ratio of carcinoma cells to hMSCs (2:1 ratio) was selected according to previously optimized conditions for high tumor implantation rate (24). After 6 h, both the inserts and lower culture wells were washed three times with PBS and then the medium was switched to DF-12 without FBS for 24 h.

Serum-deprived hMSC conditioned medium (hMSC SD-conditioned medium) were also used as indirect co-culture medium. hMSCs were cultured until they reached 80%

confluence, washed three times with PBS and incubated in DF-12 medium without FBS for 24 h. Then, SD-conditioned media were harvested by $0.22 \,\mu m$ filtration.

Serum-deprived co-culture groups (SD+co-culture) grew in the presence of hMSCs in DF-12 media without FBS for 24 h; SD-conditioned medium groups (SD-conditioned) were grown in the presence of only hMSCs SD-conditioned media for 24 h; serum-deprived control groups (SD+control) were grown in DF-12 media without FBS; and control groups (control) were grown in DF-12 media with FBS for 24 h. Every group had three wells.

Cell Counting Kit-8. The measurement of viable cell mass was performed with a Cell Counting Kit-8 (CCK8, Beyotime, Jiangsu, China) assay. Cells were first seeded in 24-well flatbottomed plates for 6 h. Next, the wells were prepared for the four different culture groups, and the cells were grown for 24 h. Cell proliferation was measured according to the manufacturer's instructions.

Cell apoptosis assay. Apoptotic cells were analyzed by flow cytometry. Four groups were cultured in different culture media for 24 h. Approximately $2x10^5$ cells were incubated with the Cell Apoptosis Assay according to the manufacturer's instructions (Beyotime). Briefly, cells were resuspended in 195 μ l 1X binding buffer containing 5 μ l Annexin V for 10 min at room temperature in the dark, and then mixed with another 190 μ l 1X binding buffer containing 10 μ l PI and incubated in an ice bath for another 10 min. After incubation, at least 10,000 cells were measured on a Beckman Coulter flow cytometer. Cells undergoing an early stage apoptosis are stained with Annexin V-FITC only, while cells at a late stage of apoptosis and necrotic cells are stained with both Annexin V-FITC and propidium iodide.

Transfection. GFP-tagged microtubule-associated protein 1 light chain 3 (GFP-MAP1LC3) was used to monitor autophagy through direct fluorescence microscopy. Cells undergoing autophagy were observed to have significant numbers of punctate GFP, while normal cells showed a primarily diffuse GFP signal. The carcinoma cells were seeded (2x10⁵ cells/ well) in 6-well plates overnight. The cells were transiently transfected using Lipofectamine 2000 Plus GFP-MAP1LC3 (Yrgene, China) according to the manufacturer's protocol. The cells were cultured for 24 h to ensure the expression of GFP-MAP1LC3, divided into the four experimental groups, and incubated for 24 h. Then, the cells were fixed in 4% paraformaldehyde for 10 min, stained with DAPI and analyzed under a Leica laser confocal microscope to measure the cells with GFP-MAP1LC3-positive dots.

Transmission electron microscopy (TEM). After treatment with different culture media for 24 h, cells were fixed in ice-cold 2.5% glutaraldehyde acid in 0.1 M PBS buffer for 2 h or longer, rinsed with PBS, postfixed in 1% osmium tetroxide with 0.1% potassium ferricyanide, dehydrated in a graded series of ethanol (30-90%) and embedded in Epon resin. Thin sections of 50-60 nm were cut and picked up on copper grids, post-stained with uranyl acetate and lead citrate and then observed using a Philips EM420 transmission electron microscopy.

Western blot analysis. At the end of the treatments, cells were lysed in cell lysis buffer (Beyotime) with 1 mM phenylmethylsulfonyl fluoride (PMSF), and the protein concentration of the lysate was quantified using a BCA protein assay kit (Beyotime). Equal amounts of protein for each sample were electrophoresed in SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% non-fat milk, the membranes were incubated with primary antibodies against MAP1LC3, Beclin-1, B-cell lymphoma/leukemia-2 (Bcl-2) (Abcam Inc.) and β -actin (Beyotime) overnight at 4°C. Then, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Beyotime) for 2 h. Bands were detected using ECL (Beyotime). Protein levels were quantitated by densitometry using Quantity One software (Bio-Rad Laboratories, Munich, Germany).

Animal studies. Male NOD/SCID mice aged 4-6 weeks were used in our study. All study protocols were approved by the Third Military Medical University Animal Care and Use Committee (Chongqing, China; no. 2011-11). All procedures were carried out in accordance with the advice and permission of the Institutional Ethics Committee. Two lines of carcinoma cells were treated with or without hMSCs for 24 h under serum-deprived medium and then resuspended as single-cell type suspensions $(1x10^6)$ or mixed with hMSCs $(5x10^5)$ in 0.1 ml PBS. Cells were injected subcutaneously into the left flank of the mice. The mice were examined each week and palpable tumors at the injection sites with a size of more than 3 mm in diameters were considered tumors. After 30 days, the mice were sacrificed and tumor volume was calculated according to the formula $V=\pi/6$ (length x width x height). Each group included 10 NOD/SCID mice.

Statistical analysis. All experiments were repeated at least three times. The statistical analyses were performed using SPSS v.13.0. The data were expressed as the means \pm SD and analyzed using the Student's t-test. The criterion for statistical significance was taken as p<0.05.

Results

Lung carcinoma cells have better tolerance with co-culture or hMSCs SD-conditioned media in serum-deprived conditions. There is evidence showing that hMSCs provide sufficient stromal support for tumor cells. Solid tumors are typically characterized by nutrient deprivation and an ischemic microenvironment as they grow. To determine whether hMSCs influence lung carcinoma cell survival, the lung cancer cell lines A549 and SPC-1 were used. The two cell lines were cultured in DMEM/F12 with or without hMSCs under serum-deprived conditions or with hMSC SD-conditioned media for 24 h. At the end of the treatment, cell viability analysis showed that hMSCs helped maintain the viability of A549 and SPC-1 under serum deprived conditions. The viability levels of A549 and SPC-1 cells in the SD+co-culture groups were higher than those in the SD+control groups (p<0.01) and similar to those of the SD-conditioned groups (p<0.01). Moreover, the viability of SD+co-culture was similar to that of the SD-conditioned groups (Fig. 1).



Figure 1. hMSCs protect the proliferation capacity and viability of lung carcinoma cells. The lung carcinoma cell lines A549 and SPC-1 were co-cultured with hMSCs using transwell co-culture systems (SD+co-culture) or treated with hMSC SD-conditioned medium (SD-conditioned) for 24 h under serum-deprived conditions. Serum-deprived control groups (SD+control) were grown in DF-12 media without FBS and control cells (Control) were grown in DF-12 media with FBS for 24 h. At the end of treatment, cell proliferation and viability were measured using the cell counting kit-8 assay. The cell viability is shown as the mean \pm SD. *p<0.05, **p<0.01.

The survival of lung carcinoma cells is attributed to the decrease in apoptosis induced by hMSCs under serum deprivation. As higher viability was observed for lung carcinoma cells in the co-cultured and SD-conditioned groups, we investigated the effects of hMSCs on the apoptosis of tumor cells, which occurs in stressful conditions. The Annexin V/PI assay was used to detect apoptosis by flow cytometry. Our findings showed that the apoptotic rate of A549 and SPC-1 cells was obviously reduced in the SD+co-culture and SD-conditioned groups compared with SD+control (p<0.01). Likewise, the apoptotic rate of A549 and SPC-1 SD+co-culture resembled that of the SD-conditioned groups (Fig. 2) (p>0.05).

Autophagy is activated by hMSCs in lung carcinoma cells under serum deprivation conditions. To determine whether autophagy is involved in the hMSC-mediated increase in lung carcinoma cell survival upon nutrient deprivation, we examined the accumulation of autophagosomes (25). After transient transfection with GFP-MAP1LC3 plasmids, A549 and SPC-1 cells were incubated in the four different experimental media for 24 h, and we then observed GFP-MAP1LC3 dots under a fluorescence microscope (Fig. 3A). Transmission electron microscopy was used to examine autophagic vacuoles (Fig. 3B). The A549 and SPC-1 cells in the SD+co-culture and SD-conditioned groups obviously have more autophagosomes than SD+control, and the SD+co-culture resembled the SD-conditioned groups (Fig. 3C). We also evaluated MAP1LC3-I and MAP1LC3-II protein levels using western blot analysis (Fig. 3D).



Figure 2. Apoptosis of lung carcinoma cells was decreased by hMSCs under serum deprivation. (A) A549 and SPC-1s were treated as detailed in Fig. 1. Apoptosis was detected by flow cytometry using the Annexin V/PI assay. (B) The apoptotic rates of A549 and SPC-1 in the four groups were quantified. Mean data (\pm SD) are shown. *p<0.05, **p<0.01.

Autophagy activated by hMSCs is involved in the tolerance of lung carcinoma cells to serum deprivation. To analyze whether autophagy is involved in the observed decrease in apoptosis, the effect of autophagy inhibition on cell viability was observed. The autophagy inhibitor 3-methyladenine (3-MA) (26) was used to block autophagy. SD+control group and SD-conditioned group were treated with DMSO or 3-MA, respectively. A549 and SPC-1 cells incubated with or without hMSC in serum deprivation lacked autophagic activity when treated with 3-MA (Fig. 4A and B). Moreover, greater numbers of apoptotic cells were observed in the 3-MA treatment groups compared to the non-3-MA-treated groups (Fig. 4C and D).

hMSCs favor tumorigenesis and growth after starvation in vivo. To investigate the effects of hMSCs on the growth of A549 cells and SPC-1 cells after starvation *in vivo*, we assessed the growth of tumor cells in NOD/SCID mice. After 2 weeks, all mice transplanted with carcinoma cells and hMSCs have palpable tumor nodules, compared to only 70 and 60% of the mice injected with carcinoma cells alone (Fig. 5A). The mean volume of tumors of the mice co-injected with hMSCs and tumor cells was dramatically larger than that of the control groups (Fig. 5B).

Protein expression changes in the tumor cells induced by the SD-hMSC-conditioned medium. To elucidate the molecular mechanism underlying the protective effects of hMSCs on tumor cells *in vitro*, we investigated the expression of Beclin-1 and Bcl-2 in tumor cells by western blot analysis. The results showed that the presence of MSCs during serum deprivation increased the levels of Beclin-1, a mammalian autophagy protein, in A549 and SPC-1 cells. In contrast, the expression of Bcl-2, an anti-apoptotic and anti-autophagy protein, was clearly reduced in the SD-conditioned groups. Both Beclin-1 and Bcl-2 had statistically significant differences in expression in the SD-conditioned groups and SD+control groups (Fig. 6A and B).



Figure 3. hMSC activated autophagy in lung carcinoma cells under nutrient deprivation. (A) After transient transfection with GFP-MAP1LC3 plasmids, A549 and SPC-1 cells were treated as indicated in Fig. 1, and GFP-MAP1LC3 dots were observed under a fluorescence microscope. (B) Representative electron microscopic images showing autophagic vacuoles (arrows). (C) The number of cells with autophagic vacuoles was quantified in 30 cells per group. *p<0.05, **p<0.01. (D) Expression of MAP1LC3-I and MAP1LC3-II as determined by western blot analysis.



Figure 4. Autophagy activated by hMSC is involved in the survival of lung carcinoma cells under serum deprivation. (A) The SD+control group and SD-conditioned group were treated with DMSO or 3-MA and GFP-MAP1LC3 was observed under a fluorescence microscope. (B) The expression of MAP1LC3-I and MAP1LC3-II, as measured by western blot analysis. (C) Apoptosis was detected by Annexin V/PI staining followed by flow cytometry. (D) The apoptotic rates of the SD+control group and SD-conditioned group treated with DMSO or 3-MA were quantified. The means (\pm SD) are shown. *p<0.05, **p<0.01.



Figure 5. MSCs favor tumorigenesis and growth after starvation *in vivo*. (A) Tumorigenesis in mice co-injected with tumor cells and hMSCs or control mice injected with tumor cells alone. (B) The mean tumor volumes of the co-injected and control groups. *p<0.05, **p<0.01.

Discussion

Solid tumors are composed of tumor cells and supportive nontumor components known as tumor stroma. There is a niche that is enriched with carcinoma cells and a subpopulation of closely associated stromal cells that control the activity of the carcinoma cells. hMSCs can be recruited to these niches and subsequently secrete various cytokines and growth



Figure 6. Protein expression changes in tumor cells induced by SD-hMSC-conditioned medium. (A) The expression of Beclin-1 and Bcl-2 in control, SD+control and SD-conditioned A549 cells. (B) The expression of Beclin-1 and Bcl-2 in control, SD+control and SD-conditioned SPC-1 cells.

factors (7). When tumors grow beyond 2 mm in diameter, carcinoma cells and stromal cells could undergo starvation due to lack of sufficient nutrients in the niche (27). Because of the close relationship between hMSCs and carcinoma cells, it is unclear how hMSCs influence the fate of carcinoma cells under nutrient deprivation conditions. In the present study, we used an SD co-culture system and SD-hMSC-conditioned medium to explore the effects of hMSCs on lung carcinoma cell lines A549 and SPC-1. This is the first time the role of hMSCs in lung carcinoma cells under serum deprivation has been addressed. We found that the viability of carcinoma cells grown with hMSCs is higher than that of cells grown without hMSCs after serum starvation for 24 h. Moreover, a marked decrease in apoptosis was observed in lung carcinoma cells that were co-cultured with hMSCs, supporting the protective role of hMSCs against apoptosis.

Autophagy is a well-established mechanism to degrade cytoplasmic proteins, macromolecules, and organelles and to provide a nutrient source to promote the survival of cells in metabolic distress. Accumulating evidence supports a role for autophagy in maintaining tumor cell survival in response to metabolic stress and hypoxia (28-31). Autophagy has been considered an indispensable physiological reaction for sustaining cell viability during starvation. A549 and SPC-1 cells were co-cultured with hMSCs during nutrient deprivation showed more autophagic cells than were observed in SD-condition groups. Less apoptosis was observed in the co-culture system. After the addition of 3-MA, a commonly used inhibitor of starvation or rapamycin-induced autophagy, apoptosis increased, concomitant with a decrease in autophagy. Moreover, cells at late stage apoptosis and necrotic cells were found in the 3-MA groups, supporting the protective role of hMSCs against apoptosis under nutrient starvation conditions. Similarly, 3-MA treatment also led to a dramatic decrease in the survival of single culture group under starvation conditions. This is similar to findings in starved HeLa cells, in which it was shown that the suppression of autophagy could promote apoptosis and caspase-3 activation (32). The indirect system used in this study indicated that this protective mechanism may involve cytokines such as growth factors, anti-apoptotic factors and TGF- β , which have been found in conditioned medium from SD-hMSCs (27).

In vivo, subcutaneous injection experiments showed that starved lung carcinoma cells that were co-injected with hMSCs exhibited stronger tumor initiation and growth than carcinoma cells that were injected alone. There are several possible explanations for this observation. First, tumor initiation and growth may be promoted by the protective role of hMSCs on lung carcinoma cell viability and apoptosis, as reported in this study. Further, there is evidence that hMSCs may differentiate into tumor stromal fibroblasts, forming a niche that favors tumor growth (33). Furthermore, hMSCs enhanced vascular endothelial growth factor (VEGF) expression in tumor cells (34). Importantly, as the tumor grows, the stromal cells and tumor could undergo starvation and stromal cells may secrete growth factors and anti-apoptotic factors that protect carcinoma cells (27). Overall, we hypothesize that SD-hMSCs may provide a protective niche for lung carcinoma cells. During tumor growth, the growth factors and anti-apoptotic factors secreted by hMSCs protect lung carcinoma cells and allow them to survive the nutrient deficient situation.

Beclin-1 is identified as mammalian autophagy gene that plays a major role in the formation of the autophagosome (35). It also interacts with anti-apoptotic multidomain proteins Bcl-2 family members, in particular Bcl-2 and its homologue Bcl-XL, whose overexpression would inhibit the autophagy-inducing activity of Beclin-1 (36-38). Blocking the interaction between Beclin-1 and Bcl-2 has been reported to enhance autophagy (39). We determined that the expression levels of Beclin-1 and Bcl-2 proteins in A549 and SPC-1 cells after inoculation. Under normal conditions, A549 and SPC-1 cells exhibited a basal expression of Beclin-1 and Bcl-2; under serum deprivation conditions, the expression of Bcl-2 was decreased and the expression of Beclin-1 increased. The addition of hMSCs resulted in a dramatic decrease in the levels of Bcl-2 and an increase in Beclin-1, which triggers autophagy as protective mechanism. Hence, we hypothesize that hMSCs could trigger autophagy by Beclin-1 without the inhibition of Bcl-2.

According to our studies, we conclude that hMSCs could promote tumor cell proliferation and reduce tumor cell apoptosis and that autophagy plays an important role in the survival of carcinoma cells. *In vivo*, earlier tumor initiation and growth were observed when tumor cells were subcutaneously injected along with hMSCs. The disruption of the Bcl-2 and Beclin-1 interaction may be involved in this autophagy protective mechanism. Taken together, this study provides preliminary exploratory research results for understanding stromal protection in the survival mechanism of lung carcinoma cells and show that the promotion of autophagy by hMSCs play an important role in tumor cell survival.

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