Human mesenchymal stem cells with adenovirus-mediated TRAIL gene transduction have antitumor effects on esophageal cancer cell line Eca-109

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The apoptotic ligand TNF-related apoptosis-inducing ligand (TRAIL) is believed to be a promising candidate for cancer gene therapy, yet gene therapy strategies to tackle this disease systemically are often impaired by inefficient delivery of the vector to the tumor tissue. Mesenchymal stem cells (MSCs) have been shown to home to tumor sites and could potentially act as a shield and vehicle for an antitumor gene therapy vector. Here, we used an adenoviral vector expressing TRAIL to transduce MSCs and studied the apoptosis-inducing activity of these TRAIL-carrying MSCs on esophageal cancer cell Eca-109. Our results showed that, in vitro, TRAIL-expressing MSCs were able to inhibit proliferation and induce apoptosis in Eca-109 cells by an MTT assay, co-culture experiments and flow cytometry analysis. In vivo, TRAIL-expressing MSCs also displayed an ability to inhibit tumor growth in an Eca-109 xenograft mouse model. Together, our findings indicated that the gene therapy strategy of MSCs-based TRAIL gene delivery has a wide potential value for improving the treatment of esophageal cancer.

Keywords esophageal cancer; gene therapy; mesenchymal stem cells; TRAIL

Received: December 30, 2013 Accepted: February 12, 2014

Introduction

The first choice for the treatment of esophageal cancer is surgical resection. The value of adjuvant or neoadjuvant radiation or chemotherapy is limited, because problems such as primary or acquired resistance against chemotherapy or side effects remain unsolved [1–3]. To date, despite the most aggressive therapy, the relative 5-year survival for esophageal cancer is dismal [4,5]. In order to make an impact on this disease, new therapeutic options are required.

The tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), as a physiological apoptosis-inducing molecule of 33 kD, belongs to the TNF super family and seems to be involved in the natural antitumoral and antiviral immunoresponse [6]. Moreover, TRAIL is also a potent and specific inducer of apoptosis in experimental tumor cells and can act as a powerful cancer-preventive agent [7,8]. Recombinant TRAIL together with bortezomib has been used for treating esophageal cancer. Gene therapy approaches utilizing TRAIL have also been shown to be effective in experimental tumor models [9,10]. In addition, due to either partial or complete resistance to TRAIL-mediated apoptosis in some tumor cell lines, many researchers have explored how this resistance might be overcome in order to improve the clinical outcome. Combination therapies involving several conventional and novel chemotherapeutic drugs have been reported to increase TRAIL-mediated apoptosis in these resistant cells. For example, the bortezomib could enhance the susceptibility to TRAIL in tested esophageal squamous cell carcinoma cell lines [11].

Bone marrow derived mesenchymal stem cells (MSCs), a kind of stromal cells that reside within the adult bone marrow, can self-renew and differentiate into mesodermal cell lineages (osteocytes, adipocytes, chondrocytes, myocytes, cardiomyocytes, fibroblasts, myofibroblasts, epithelial cells, and neurons), support hematopoiesis and suppress graft-versus-host disease [12–14]. It has been controversial whether MSCs themselves could inhibit the growth of tumor cells in vitro and in vivo. However, most studies have shown that MSCs had an ability to inhibit the proliferation of tumor cells in vitro [15]. In addition, it has also been found that MSCs have an ability to migrate to and incorporate within the stroma of tumors [16]. Recently, this property of MSCs has begun to show promise as a potential delivery vector to direct targeting antitumor agents to the experimental tumor cells and their tumor models. Moreover, MSCs transduced with a lentivirus or adenovirus vector expressing TRAIL have also been shown to be effective in many of experimental tumor models [17–20].

In this study, we investigated the effect of TRAIL-expressing MSCs with adenoviral vectors on esophageal...
cancer cell line Eca-109 in vitro and in vivo, which will provide a new effective approach for the treatment of esophageal cancer.

Materials and Methods

Cell culture

Human esophageal cancer cell line Eca-109 and human embryonic kidney cells 293T, from Institute of Biochemistry and Cell Biology (Shanghai, China), were separately cultured in RPMI1640 and DMEM supplemented with 10% fetal bovine serum (FBS; Gibco, Gaithersburg, USA), 100 U/ml penicillin and 100 mg/ml streptomycin. Human MSCs (Cyagen, Chicago, USA) were derived from bone marrow of healthy adults and cultured in human MSC growth medium supplemented with 10% fetal calf serum (Cyagen). MSCs before the sixth subculture were used in the experiments.

Construction of TRAIL-modified MSCs

The adenovirus carrying TRAIL (Ad.TRAIL) was constructed by using the replication-defective adenoviral vectors (Microbix, Inc., ON, Canada) with deletions of the E1 and E3 regions and the secretable trimeric form of the full-length TRAIL gene inserted in E1 under the control of the cytomegalovirus promoter. The adenovirus was produced by transfecting 293T cells and purified by double banding on CsCl gradients. The adenovirus was concentrated by ultracentrifugation at 29,700 g at 4°C and stored at −80°C before use. Human MSCs were transduced with the virus at a multiplicity of infection (MOI) of 1000 pfu/cell. TRAIL expression of the transduced MSCs was verified by enzyme-linked immunosorbent assay (ELISA) and western blot analysis.

ELISA

When the transduced MSCs were separately cultured for 24, 48, and 72 h, 1 ml of the supernatants were collected to measure the content of TRAIL protein using an ELISA kit (R&D Systems, Minneapolis, USA) according to the manufacturer’s instructions.

Western blot analysis

MSCs in the logarithmic growth phase were harvested by trypsinization and lysed in cell lysis buffer. The lysates were centrifuged at 14,000 g and the supernatants were collected. Samples were subject to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. After being blocked with 4% skim milk in Tris-buffered saline, the membrane was incubated with primary antibody against TRAIL or anti-actin (Santa Cruz, Santa Cruz, USA) overnight at 4°C, followed by incubation with a peroxidase conjugated secondary antibody. Detection was performed using an ECL kit (Sigma, St. Louis, USA).

Co-culture of MSCs and Eca-109 cells

Eca-109 cell suspensions (1 ml, 5 x 10⁵ cells) and TRAIL-expressing MSCs (Ad.TRAIL-MSCs) cell suspensions (1 ml, 1 x 10⁵ cells), or the control unmodified MSCs and MSCs transduced with only adenovirus (Ad.MSCs) cell suspensions (1 ml, 1 x 10⁶ cells), were respectively mixed and cells were cultured together for 48 h. Then the morphology of Eca-109 cells was observed.

MTT assay

When the unmodified MSCs and transduced MSCs were 80%–90% confluent in the culture flask, the medium was collected separately, filtered through a 0.2-mm filter, and used as the conditioned medium. Eca-109 cells were inoculated in the tumor cell culture medium supplemented with 50% conditioned medium using the 96-well plates at a density of 5 x 10³ cells per well. After 24, 48, 72, or 96 h of incubation, 3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) was added and incubated for another 4 h. After formazan products were solubilized with dimethyl sulfoxide, the optical density was measured at 490 nm and cell growth curves were generated.

Flow cytometry analysis

After being cultured in the mixture of RPMI 1640 medium and the conditioned medium (1 : 1) for 48 h, Eca-109 cells were collected by digesting with 0.25% trypsin without ethylenediamine tetraacetic acid (EDTA) for apoptosis analysis using annexin V-FITC cell apoptosis detection kit (KeyGEN, Nanjing, China) according to the manufacturer’s instructions. Cell apoptosis was detected by flow cytometry (FACSCalibur; BD Pharmingen, Franklin Lakes, USA). Data were analyzed by the software WinMDI2.9.

Animal experiments

Six-week-old female BALB/c nude mice were from the Institute of Zoology, Chinese Academy of Sciences (Beijing, China). Eca-109 cells, as single-cell type suspensions [5 x 10⁶ cells in 0.1 ml phosphate buffered saline (PBS)], were injected subcutaneously at the same site of the back of the mice. All procedures were carried out in accordance with the advice and permission of the Institutional Ethical Committee of Shandong University. After two weeks, the tumors were directly injected with 1 x 10⁶ Ad.TRAIL-MSCs (1000 pfu/cell), Ad-MSCs (1000 pfu/cell) or unmodified MSCs, respectively. Tumors were measured once a week and tumor volume was calculated, following over 2 weeks.
Statistical analysis
Data were expressed as mean ± standard deviation (SD) from at least three independent experiments by SPSS software. Statistical differences between different test conditions were determined using Student’s t test. \( P < 0.05 \) was regarded as statistically significant.

Results

TRAIL-expressing MSCs inhibit proliferation and induce apoptosis in Eca-109 cells in vitro
Human MSCs were transduced with the virus at an MOI of 1000 pfu/cell to produce almost 100% successful transduction. MSCs were examined for TRAIL expression using western blot analysis and ELISA. Western blot analysis showed that TRAIL protein expression in Ad.TRAIL-MSCs was obvious; however, it was not found in Ad.MSCs or unmodified MSCs (Fig. 1A). ELISA results showed that the content of TRAIL protein in the supernatants of Ad.TRAIL-MSCs cultured for 24, 48 and 72 h were \( 11.87 ± 2.66 \), \( 33.31 ± 3.52 \), and \( 27.72 ± 2.68 \) ng/ml, respectively (Fig. 1B), and TRAIL protein in the supernatants of MSCs and Ad.MSCs was not found either.

The co-culture experiments showed that Eca-109 cell growth was inhibited by TRAIL-expressing MSCs. Due to the co-culture with Ad.TRAIL-MSCs, cell density and adherent rate of Eca-109 declined, and cell process of most Eca-109 cells disappeared. However, cell morphology of Eca-109 after co-culture with MSCs and Ad.MSCs did not change obviously (Fig. 2). MTT assay also showed an obviously inhibiting effect on the proliferation of Eca-109 cells when treated with the conditioned medium from Ad.TRAIL-MSCs, compared with treatment with the conditioned medium from MSCs and Ad.MSCs (\( P < 0.01 \); Fig. 3).

To investigate the effect of TRAIL-expressing MSCs on the apoptosis of esophageal cancer cell Eca-109, we tested the apoptotic ratios of Eca-109 cells treated with the conditioned medium by flow cytometric analysis. Our results showed that the apoptotic ratios of Eca-109 cells treated with the conditioned medium from MSCs, Ad.MSCs and Ad.TRAIL-MSCs were \( 11.60% ± 1.19% \), \( 10.51% ± 0.96% \), and \( 38.62% ± 1.87% \), respectively. Compared with control groups, TRAIL-expressing MSCs could significantly induce the apoptosis of Eca-109 cells (\( P < 0.001 \); Fig. 4).

TRAIL-expressing MSCs inhibit tumor growth of Eca-109 xenografts in mice
To examine whether MSCs transduced with TRAIL would have antitumor activity in vivo, we injected \( 5 \times 10^6 \) of Eca-109 cells into nude mice (\( n = 8 \)). After 2 weeks, the tumors had reached an average volume of \( \sim 180 \text{ mm}^3 \). Then, \( 1 \times 10^6 \) MSCs, Ad.MSCs, or Ad.TRAIL-MSCs were injected into the tumor mass. Two weeks after injection, the
mean volume of tumors of the mice injected with Ad.TRAIL-MSCs was dramatically lower than those of control groups ($P < 0.01$; Fig. 5A). Moreover, necrosis in tumor mass occurred in half ($n = 4$) of the mice injected with Ad.TRAIL-MSCs, but not in tumor mass of the control groups (Fig. 5B).

**Discussion**

At present, cancer gene therapy has long been seen as the hope of successful treatment for metastatic cancer. However, due to the lack of efficient vectors, many research on this strategy have not achieved perfect results. For example, many previous adenovirus-based strategies have been hampered by the innate and adaptive immune response and inaccessibility of some of the tumor sites. MSCs have been shown to home to tumor sites and could potentially act as a shield and vehicle for a tumoricidal gene therapy vector [17–19]. The ability of TRAIL to lead to tumor cell apoptosis with no effect on normal cells makes it an extremely exciting molecule for tumor therapy [8]. Due to their dominant positions, recently, many researchers have explored the feasibility of gene therapy using MSCs as a vector and TRAIL as a target gene in different experimental tumor models. Mohr et al. [17] demonstrated the potential therapeutic use of TRAIL-expressing MSCs in lung cancer cells and the stability of this vector in the context of the blood environment. Their results showed that TRAIL-carrying MSCs had apoptosis-inducing activity on A549 lung carcinoma cells in a xenograft mouse model. In addition, transducing MSCs with viral DNA to transgenic protein production was not inhibited by exposure of MSCs to human serum containing high levels of adenovirus neutralizing antibodies. Moreover, Ad.TRAIL-transduced MSCs could also induce apoptosis of A549 cells in the presence of physiological concentrations of white blood cells (WBCs), erythrocytes, and sera from human donors that inhibit or neutralize adenovirus [17]. Loebinger et al. [19] showed that TRAIL-expressing MSCs were able to home to and kill cancer cells, and significantly reduce tumor growth in a lung metastatic cancer model of breast cancer cell MDAMB231. In addition, TRAIL-expressing MSCs were also confirmed to have antitumor ability in other experimental tumor models of cancer cells, such as U87 glioma cell and colorectal cancer HCT116 cell [18,21].

In this study, we showed that MSCs could be engineered to express TRAIL to induce the apoptosis of esophageal cancer cells Eca-109. Our findings revealed that TRAIL-expressing MSCs were able to inhibit proliferation and induce apoptosis of Eca-109 cells in vitro and reduce the tumor growth of Eca-109 xenografts in mice. Esophageal cancer, one of the most lethal human tumors, is the fourth leading cause of cancer death in China, for which incidence is also increasing.

**Figure 3. The effect of TRAIL-expressing MSCs on Eca-109 cell proliferation measured by MTT assay** The lowest growth curve represents the proliferating activity of Eca-109 cells treated with the supernatants of Ad.TRAIL-MSCs (C group); the superior two were the growth curves of control groups (A and B groups: MSCs and Ad.MSCs). The absorbance was measured everyday in a period of 4 days. The results were expressed as mean ± SD of three experiments. *$P < 0.01$.

**Figure 4. The effect of TRAIL-expressing MSCs on Eca-109 cell apoptosis detected by flow cytometric analysis in vitro** The figures showed the apoptosis of Eca-109 cells treated respectively with the conditioned medium from MSCs (A), Ad.MSCs (B), and Ad.TRAIL-MSCs (C). The figures are the representative of three experiments.
in the western world. In many of the patients with esophageal cancer, the tumors are detected at an advanced stage. Despite improvements in perioperative treatment and surgical techniques, rapid recurrence led to the death of many patients with advanced esophageal cancer. Conventional treatments including surgical resection, radiotherapy, chemotherapy, or their combinations rarely result in long-term survival for advanced cancer patients [22]. Efforts are now focused on the multimodality treatments including biological therapy and gene therapy, in an attempt to improve local control and eliminate micrometastases. Our data suggested that Ad.TRAIL-transduced MSCs may thus be a potentially effective therapeutic agent for advanced esophageal cancer.

**Funding**

This work was supported by the Natural Science Foundation of Shandong Province (ZR2009CM090).

**References**


