Interleukin-1α induces immunosuppression by mesenchymal stem cells promoting the growth of prostate cancer cells

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Abstract. Mesenchymal stem cells (MSCs) are generally used in tissue engineering, regenerative medicine and therapy for immune disorder disease. MSCs are also employed as drug carriers for tumor therapy due to their ability to migrate to tumor tissue. However, due to the immunosuppressive function of MSCs, the application of MSCs in prostate cancer therapy remains limited. In this study, we investigated the underlying mechanism by which MSCs enable prostate cancer cells to escape from immune surveillance in the inflammatory microenvironment. Firstly, we demonstrated that compared with the control groups, MSCs pretreated with IL-1α effectively promoted the growth of the mouse prostate cancer cell line RM-1 in vivo. Furthermore, when RM-1 prostate cancer cells were co-injected with MSCs pretreated with IL-1α, tumor incidence significantly increased in allogeneic recipients. In addition, we investigated the mechanism through which MSCs promote the ability of RM-1 cells to escape from immune injury. The results revealed that IL-1α led to the upregulation of TGF-β in MSCs. The inflammatory cytokine-induced promotive effect of MSCs on RM-1 cells in vivo was inhibited by TGF-β siRNA. The results of our study suggest that inflammatory cytokines induce the immunosuppressive function of MSCs which enables prostate cancer cells to escape from immune injury.

Introduction

Mesenchymal stem cells (MSCs), which originate from the mesodermal germ layer, are a subset of non-hematopoietic stem cells that exist in the bone marrow (1,2). MSCs have been described as an adherent, fibroblast-like population and have the ability to differentiate into multiple lineages, including chondrocytes, osteocytes, adipocytes, myocytes and astrocytes, and are a potential source of stem cells for cellular and genetic therapy (3,4). MSCs also exist in other tissues, including adipose tissue, umbilical cord, fetal liver, muscle and lung (3,5-8). MSCs are capable of expanding more than 104-fold in culture without loss of their multilineage differentiation potential.

The immunosuppressive function of MSCs has been reported in several studies (9-12). Koç et al reported that when allogeneic MSCs were infused along with allogeneic bone marrow into patients with metachromatic leukodystrophy or Hurler’s syndrome, there was no evidence of alloreactive T cells and no incidence of GvHD (13). MSCs have also been used to prevent or treat autoimmune diseases, including experimental autoimmune encephalomyelitis and collagen-induced arthritis (14,15).

MSCs have a tropism for tumors (16) and several studies have reported contradictory results concerning the effect of MSCs on tumor growth. Hall et al (17) demonstrated that the co-culturing of ALL cell lines with VCAM-1-overexpressing stromal cells enhanced the survival of the leukemic cells in a PI-3 kinase-dependent manner, compared with co-culturing with stromal cells expressing only endogenous VCAM-1. Djouad et al (18) revealed that MSCs exhibit side effects related to systemic immunosuppression that favor induced tumor growth in vivo. Conversely, MSCs have been reported to be anti-tumorigenic in a mouse model of Kaposi’s sarcoma by inhibiting AKT activity (19). Tumorigenesis is always associated with chronic inflammation. Therefore, it is essential to observe the effects of MSCs on tumor growth in an inflammatory environment.

In this study, we used the RM-1 prostate cancer cell line to investigate the effect of MSCs on tumor growth in an inflammatory environment. The incidence and development of prostate cancer is often accompanied by an inflammatory microenvironment. Therefore, it is important to determine the mechanism of the inflammatory cytokine-induced immunosuppressive effect of MSCs in prostate cancer cells.

Materials and methods

Reagents. Recombinant mouse IL-1α was from Peprotech, Inc. (La Jolla, CA, USA). Anti-mouse CD34, CD45, CD90,
CD105 and CD29 antibodies were from eBioscience (San Diego, CA, USA).

Cells and animals. MSCs were generated from bone marrow flushed out of the tibias and femurs of 4-6-week-old mice. The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Non-adherent cells were removed after 72 h and adherent cells were maintained with medium replenishment every 3 days.

The murine prostate cancer line RM-1 was cultured at 37°C with 5% of CO₂ in RPMI-1640 with 10% FBS, supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were subcultured every 3 days when they reached 70-80% confluence.

Male Balb/c and C57BL/6 mice, 6-8 weeks old, were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences, Shanghai, China. Mice were housed in pathogen-free conditions and all procedures were performed according to the guidelines of the Committee on Animals of the Chinese Academy of Sciences.

Multi-differentiation of MSCs. MSCs were cultured with an osteoinductive medium consisting of DMEM supplemented with 10% FBS, β-mercaptoethanol, 100 µM L-ascorbic acid, 10 nM dexamethasone and 10 mM β-glycerophosphate for 14 days. The cells were then stained with alizarin red to reveal calcium deposition, characteristic of osteoblasts. MSCs were induced to differentiate into adipocytes by culturing with DMEM supplemented with 0.5 mM isobutylmethylxanthine, 60 µM indomethacin, 10 nM dexamethasone and 10 µg/ml insulin for 14 days. The formation of adipocytes was verified by staining for triglycerides with Oil red O to detect intracellular lipid accumulation.

RM-1 murine prostate cancer model. RM-1 cells and MSCs were prepared either as single-cell type suspensions (1×10⁶ cells in 200 µl PBS) or as a mixture of cells (1×10⁶ RM-1 cells and 2×10⁵ MSCs in 200 µl of PBS). RM-1 cells (alone or mixed with MSCs) were subcutaneously administered in the armpit area of Balb/c or C57BL/6 mice. Tumor incidence was evaluated 3 times per week.

Mixed lymphocyte reaction (MLR). Mouse spleens were disaggregated into 10 ml RPMI-1640 medium to isolate splenocytes. Erythrocytes were lysed with 0.8%/4% NH₄Cl and subsequently washed 3 times with RPMI-1640. Trypan blue dye exclusion was used to assess cell count and viability. Splenocytes were incubated with 5 µg/ml concanavalin A (ConA; Sigma-Aldrich, St. Louis, MO, USA) for 72 h and then cultured with IL-2 (200 U/ml) for proliferation. Splenocyte cultures were maintained in RPMI-1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 mM of β-ME (complete medium). MSCs were added to the MLR to provide a 200-µl final volume. Following 3 days of incubation, 1 µCi/well (0.037 MBq/well) [³H]-thyidine was added overnight and thymidine incorporation was measured using a β-scintillation counter. The data were presented as the percentage of the relative proliferative response, corresponding to the mean counts per min (cpm) of a responder stimulator pair in the absence of MSCs which was attributed a 100% value.

Real-time PCR. MSCs were incubated with IL-1α (20 ng/ml) for 12 h and the total cell mRNA was collected with TRIzol reagent (Invitrogen). cDNA was synthesized using M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA) and 2 µg total RNA and oligo dT18-primers. PCR amplification was carried out using 2-µl aliquots of cDNA. Real-time PCR was performed in triplicate using the SYBR PrimeScript RT-PCR kit (Takara Bio, Inc., Shiga, Japan). The primer sequences for TGF-β were: forward, 5′-TGTCACCGGATTTGTCCGC-3′; reverse, 5′-CTCGCGCCCGTGATGAA-3′. Total sample RNA was normalized to endogenous β-actin mRNA. Thermocycler conditions included an initial hold at 50°C for 2 min and then 95°C for 10 min, followed by a two-step PCR program of 95°C for 15 sec and 60°C for 60 sec repeated for 40 cycles using an Mx 4000 system (Stratagene, La Jolla, CA, USA) on which data were collected and quantitatively analyzed. Expression levels of mRNA were presented as fold changes relative to an untreated control.

Western blot analysis. Cells were washed with PBS solution, and protein was then extracted according to an established protocol. Furthermore, proteins were mixed with Laemmli sample buffer, heated at 65°C for 10 min, loaded (20 µg for each sample), separated by sodium dodecyl sulfate-polyacrylamide gel (7.5%) electrophoresis under denaturing conditions and electroblotted onto nitrocellulose membranes. The nitrocellulose membranes were blocked by incubation in blocking buffer (1% BSA in Tris-buffered saline-0.1% Tween 20), incubated with anti-TGF-β-antibody (Abcam, Cambridge, UK), and washed and incubated with anti-rabbit peroxidase-conjugated secondary antibody (Invitrogen). Signals were visualized by chemiluminescent detection. Blots were quantified using Quantity One software from Bio-Rad (Hercules, CA, USA), and TGF-β expression was normalized to values in the control group. Equal loading of samples was verified by Coomassie blue staining of simultaneously run gels. Gels were run 4 times and the images shown are representative.

Statistical analysis. Statistical analysis of the data was performed using GraphPad Prism 4 software. The Student's
The t-test was used to compare the mean values of the two groups. Data among 3 or more groups were compared using the one-way analysis of variance, followed by the Dunnett’s post hoc test. Final values were expressed as the mean ± SEM. P<0.05 was considered to indicate a statistically significant result.

Results

Pretreatment of MSCs with inflammatory cytokines promotes the growth of RM-1 prostate cancer cells in vivo. We identified long spindle-shaped fibroblastic cells isolated from bone marrow by examining their surface markers and ability to differentiate. The results demonstrated that these cells were positive for CD90, CD105 and CD29 and negative for CD34 and CD45. Furthermore, these cells differentiated into adipocytes and osteoblast-like cells. The results indicated that the cells isolated from the bone marrow had properties that were consistent with those of MSCs (Fig. 1A and B).

MSCs, which were either pretreated with inflammatory cytokines IL-1α or not, were co-injected with RM-1 cells into Balb/c mice. We found that a more rapid growth of the RM-1 cells that were mixed with non-pretreated MSCs in vivo than that of the RM-1 cells alone. Compared with the control group, MSCs pretreated with IL-1α demonstrated a tumor-promoting effect (Fig. 2). Conditioned media were collected from the MSCs and IL-1α-pretreated MSCs. The conditioned media were infused into subcutaneous RM-1 tumor-bearing mice via tail vein injection. Compared with the control groups, the conditioned medium from IL-1α-pretreated MSCs significantly enhanced the tumor growth in vivo (Fig. 2). These results indicate that IL-1α stimulates MSCs to promote the growth of RM-1 tumors in vivo.
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RM-1 prostate cancer cells are not rejected by C57BL/6 mice when co-injected with MSCs. To determine the immunosuppressive effect of MSCs in the promotion of tumor growth in vivo, we combined RM-1 cells with MSCs, which were either pre-incubated with IL-1α or not, and implanted subcutaneously in C57BL/6 mice. The RM-1 cells developed into tumors when implanted in Balb/c mice; however, these cells were rejected by the C57/BL6 mice. When RM-1 cells were co-injected with MSCs, the tumor incidence markedly increased. Furthermore, compared with the control group, IL-1α-pretreated MSCs further enhanced the tumor incidence (Fig. 3). In addition, we found that the only conditioned medium able to increase the RM-1 tumor incidence in the C57/BL6 mice was that obtained from IL-1α-pretreated MSCs (Fig. 3). Taken together, the results suggest that IL-1α induces the immunosuppressive action of the MSCs, which may help the RM-1 cells to escape from immune rejection by the C57BL/6 mice.

IL-1α induces the immunosuppressive function of MSCs by upregulating TGF-β. We employed the MLR to examine the immunosuppressive function of MSCs induced by IL-1α. We activated the splenocytes from Balb/c mice with Con A for 72 h and IL-2 (20 ng/ml) was added to maintain proliferation. Splenocytes (1x10^5/well) were co-cultured with MSCs at a ratio of 10:1 in a 96-well plate for 72 h. Conditioned media, collected from the MSCs, were added to the splenocyte culture system and the proliferation of the splenocytes was observed for 72 h. An 3H-incorporation assay was employed to assess the proliferation of the splenocytes (*P<0.05). MSCs, mesenchymal stem cells; cpm, counts per min.

**Figure 3.** RM-1 prostate cancer cells are not rejected by C57BL/6 mice when co-injected with MSCs. C57BL6 mouse MSCs (2x10^5) were pretreated with IL-1α (20 ng/ml) for 12 h, mixed with RM-1 cells (1x10^6) and then subcutaneously administered in the armpit area of C57BL6 mice. In addition, the conditioned media CM1, from MSCs, and CM2, from IL-1α-pretreated MSCs, were collected. The conditioned media were infused via the tail vein once every 3 days into Balb/c mice that had been implanted with RM-1 cells. Tumor incidence was observed to evaluate the immunosuppressive function of the MSCs that assisted the RM-1 cells to escape from immunological rejection by the Balb/c mice. As negative controls, RM-1 cells or MSCs alone were implanted in the C57/BL6 mice. MSCs, mesenchymal stem cells; CM1, conditioned medium 1; CM2, conditioned medium 2.

**Figure 4.** IL-1α induced the immunosuppressive function of MSCs. Balb/c mouse MSCs were pretreated with IL-1α (20 ng/ml) for 12 h. Balb/c splenocytes were activated by incubation with ConA (5 µg/ml) for 72 h and IL-2 (20 ng/ml) was added to maintain proliferation. Splenocytes (1x10^5/well) were co-cultured with MSCs at a ratio of 10:1 in a 96-well plate for 72 h. Conditioned media, collected from the MSCs, were added to the splenocyte culture system and the proliferation of the splenocytes was observed for 72 h. An 3H-incorporation assay was employed to assess the proliferation of the splenocytes (*P<0.05). MSCs, mesenchymal stem cells; ConA, concanavalin A; cpm, counts per min.

**Figure 5.** Immunosuppressive function of MSCs was induced by IL-1α in a TGF-β-dependent manner. Balb/c mouse MSCs were pretreated with IL-1α (20 ng/ml) for 12 h. (A) Real-time PCR was employed to examine the expression levels of TGF-β mRNA in the MSCs. (B) Western blotting was used to detect the expression levels of TGF-β protein. (C) Splenocytes were co-cultured with MSCs with or without TGF-β siRNA. Cell proliferation was examined by a 3H-incorporation assay after 72 h (*P<0.05). MSCs, mesenchymal stem cells; cpm, counts per min.
in Fig. 5A and B, IL-1α effectively upregulated the expression of TGF-β in the MSCs. To confirm the role of TGF-β in the immunosuppressive function of the MSCs, TGF-β siRNA was used to inhibit the expression of TGF-β in the MSCs. In mixed co-cultures of splenocytes and MSCs pre-stimulated by IL-1α, splenocyte proliferation was restored to normal levels by TGF-β siRNA (Fig. 5C). These results suggest that TGF-β is the key factor that mediates the IL-1α-induced immunosuppressive effect of the MSCs on splenocyte proliferation.

Enhancement of RM-1 cell growth in vivo by MSCs was prevented by TGF-β siRNA. We have demonstrated that IL-1α effectively induces the ability of MSCs to promote the growth of RM-1 cells in vivo and that this enhancement may be associated with the immunosuppressive function of MSCs. We have also shown that the IL-1α-induced immunosuppressive action of the MSCs was mediated by TGF-β. Therefore, we used TGF-β siRNA to confirm the role of TGF-β in the immunosuppressive effect of the MSCs. The results showed that the promotive effect of MSCs on tumor growth in vivo induced by IL-1α was inhibited by TGF-β siRNA (Fig. 6A). Furthermore, the enhancement of RM-1 tumor incidence in the C57/BL6 mice by IL-1α-pretreated MSCs was reduced following the use of TGF-β siRNA (Fig. 6B). These data suggest that TGF-β is a key factor in the immunosuppressive action of MSCs that enables RM-1 cells to escape from immune injury.

Discussion

It has been reported that MSCs are able to differentiate into osteoblasts, chondrocytes, adipocytes, myotubes, neural cells and hematopoietic supporting stroma (3,4,20). MSCs have been recognized to contribute to the regeneration of a wide variety of organs and to the healing of certain diseases (21-23). Furthermore, MSCs also are influential in the treatment of various degenerative diseases and immune disorders. Therefore, MSCs have been regarded as a potential therapy for numerous diseases. However, the immunosuppressive effects of MSCs have been reported in several studies (9-12), and in certain circumstances, the immunosuppressive effect may promote tumor growth. Therefore, it is essential to observe the effect of MSCs on tumor growth in an inflammatory environment.

In this study, we investigated the underlying mechanism by which MSCs enable prostate cancer cells to escape from immune surveillance in the inflammatory microenvironment. Firstly, we demonstrated that in comparison to the control groups, MSCs pretreated with IL-1α effectively promoted the growth of the mouse prostate cancer cell line RM-1 in vivo. Furthermore, when RM-1 prostate cancer cells were co-injected with MSCs pretreated with IL-1α, tumor incidence significantly increased in allogeneic recipients. In addition, we investigated the mechanism by which MSCs enable RM-1 cells...
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Several studies have demonstrated that MSCs are able to
promote tumor growth. Hall et al have shown that the
coculturing of ALL cell lines with VCAM-1-overexpressing
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Djouad et al revealed that MSCs exhibited side effects related
to systemic immunosuppression which induced tumor growth
in vivo (18). Conversely, MSCs have been reported to be
anti-tumorigenic in a mouse model of Kaposi's sarcoma by
inhibiting AKT activity (19). Liu et al have shown that IFN-γ
and TNF-α are able to induce the upregulation of VEGF in
MSCs, which may be a significant mechanism for the promotion
of tumor growth (24). The results of our study suggest that
inflammatory cytokines, including IL-1α, are key factors that
regulate the actions of MSCs on tumor growth.

There are still a number of problems limiting the application
of MSCs in clinical therapy, particularly the regulatory
effect of the microenvironment. Therefore, it is necessary to
investigate the biological activity of the MSCs in combination
with the microenvironment, in order to improve the clinical
application of MSCs in tissue engineering and regenerative
medicine. Our results suggest that inflammatory cytokines,
including IL-1α, are key factors that induce the immunosuppressive
activity of MSCs and enable the tumor cells to
 evade immune surveillance. Therefore, the use of MSCs in
cancer therapy should be carried out with caution.

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