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Effect of TGF-β1 Stimulation on the Secretome of Human Adipose-Derived Mesenchymal Stromal Cells

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Abstract

Adipose tissue is an attractive source of mesenchymal stromal cells (MSCs) owing to the relative ease of obtaining large volumes with more MSC abundance compared with other sources. Increasing evidence supports the fact that trophic factors secreted by MSCs play a pivotal therapeutic role. Several strategies in regenerative medicine use MSCs, mainly exploiting their immunosuppressive effect and homing capacity to sites of damage. Transforming growth factor-β1 (TGF-β1) is a pleiotropic cytokine that, depending on the cell niche, can display either anti-inflammatory or proinflammatory effects. TGF-β1 expression increases in various tissues with damage, especially when accompanied by inflammation. Thus, we analyzed the effect of TGF-β1 on the secretion by adipose-derived mesenchymal stromal cells (ASCs) of a panel of 80 cytokines/chemokines using an antibody array. To avoid a possible effect of fetal bovine serum (FBS) on ASCs secretion, we performed our analysis by culturing cells in FBS-free conditions, only supplemented with 0.1% of bovine serum albumin. We report the cytokine profile secreted by ASCs. We also found that TGF-β1 exposure modulates 8 chemokines and 18 cytokines, including TGF-β1 and -β2, and other important cytokines involved in immunosuppression, allergic responses, and bone resorption. STEM CELLS TRANSLATIONAL MEDICINE 2015;4:894–898

Significance

Mesenchymal stromal cells (MSCs) secrete a broad spectrum of bioactive macromolecules that are both immunoregulatory and serve to structure regenerative microenvironments in fields of tissue injury. Increases or decreases in the production of TGF-β1 have been linked to numerous disease states, including autoimmune diseases and cancer. The secretome of MSCs stimulated with TGF-β1 is largely unknown. Thus, the present study makes an important contribution toward a better understanding of how MSCs could be affected by a cytokine normally upregulated in various diseases.

Introduction

Mesenchymal stromal cells (MSCs) have great potential in regenerative medicine, and evidence is accumulating that the therapeutic benefits of MSCs are largely dependent on their secretion of trophic factors. Many of them are critical mediators in angiogenesis and the prevention of cell apoptosis and immunosuppression (reviewed in [1]).

Transforming growth factor (TGF)-β is a multifunctional cytokine, involved in critical processes such as embryonic development, cell maturation and differentiation, wound healing, and immune system regulation [2]. It maintains immune homeostasis by acting as a potent immune suppressor through inhibition of proliferation, differentiation, and activation of immune cells. Paradoxically, and depending on the cell microenvironment, TGF-β can also display proinflammatory

properties [2]. It has been reported that its expression increases in various tissues with damage, especially when accompanied by inflammation [3]; therefore, TGF-β has become a promising target for the treatment of cancer, fibrosis, asthma, and autoimmune diseases [2].

The cytokine secretion profile of MSCs derived from different organs has recently been investigated [4–7]. However, the effect of TGF-β1 on the MSC secretome remains largely unknown. In the present study, we show the secretion profile of adipose-derived MSCs (ASCs) in fetal bovine serum (FBS)-free conditions, in both the presence and the absence of TGF-β1 stimulation. In conditioned medium of ASCs, we identified a set of cytokines/chemokines sensitive to TGF-β1 stimulation and mainly involved in allergic responses, T-cell immunosuppression, and bone remodeling.

MATERIALS AND METHODS

Isolation, Culture, and Differentiation of ASCs

Subcutaneous adipose tissue was obtained from healthy female donors undergoing elective surgical procedures (age 30–40 years) at the Plastic, Aesthetic and Reconstructive Surgery Department (Hospital Italiano, La Plata, Argentina), after approval by the local ethical board and providing voluntary written informed consent. Human ASCs were isolated and cultured as described previously [8]. For the evaluation of the differentiation capacity of ASCs, the cells were seeded at a concentration of 7.5×10^3 to 1×10^4 cm^{-2} in a 24-well plate and incubated with osteogenic or adipogenic medium, as described previously [9].

TGF-β1 Stimulation and Cytokine/Chemokine Analysis

Equivalent numbers of ASCs were seeded in complete medium, and after 6–8 hours, the cells were starved for 16 hours in serum-free medium. The cells were treated with 3 ng/ml (0.12 nM) TGF-β1 for 72 hours in Dulbecco's modified Eagle's medium supplemented with 0.1% bovine serum albumin (BSA), 1% penicillin/streptomycin, and 1% glutamine (Gibco, Life Technologies, Carlsbad, CA, http://www.lifetechnologies.com). The cells not stimulated with TGF-β1 were treated using the same method. The supernatants were filtered and frozen after collection.

The cytokine/chemokine array kit G5 (Ray Biotech, Norcross, GA, http://www.raybiotech.com) was used to detect a panel of 80 proteins in cell supernatants in accordance with the manufacturer's instructions. The arrays were analyzed using a Typhoon 9410 Variable mode Imager (GE Healthcare Life Sciences, Piscataway, NJ, http://www.gelifesciences.com). The signal intensity values were measured using the image analysis software Image-Quant TL, version 7.0 (GE Healthcare Life Sciences). The data were analyzed using the RayBio Antibody Array Analysis Tool (Ray Biotech).

RNA Isolation and Reverse Transcription-Quantitative Polymerase Chain Reaction

Total RNA was obtained using the SV Total RNA Isolation System and cDNA generated with Moloney murine leukemia virus reverse transcriptase according to the specifications stated by the manufacturer (Promega Corp., Madison, WI, http://www.promega.com). To measure the mRNA levels of TGF-β receptor type II splice variants (TβRIIA and TβRIIB), reverse transcription-quantitative polymerase chain reaction was performed using FastStart Universal SYBR Green Master (Rox) (Roche Applied Science, Mannheim, Germany, http://www.roche-applied-science.com), and the following primers: TβRIIA forward, 5′-GCACGTTCAGAAGTCGGT-TAA-3′; TβRIIB forward, 5′-CTGTAATAGGACTGCCCATC-3′; and TβRIIA and TβRIIB reverse, 5′-TCTCTAGTGTTATGTTCTCGTC- 3′. The results were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels.

RESULTS

Human ASCs were isolated and cultured according to a previously described protocol [10]. The differentiation potential of ASCs was established by incubation in adipogenic and osteogenic medium (Fig. 1).
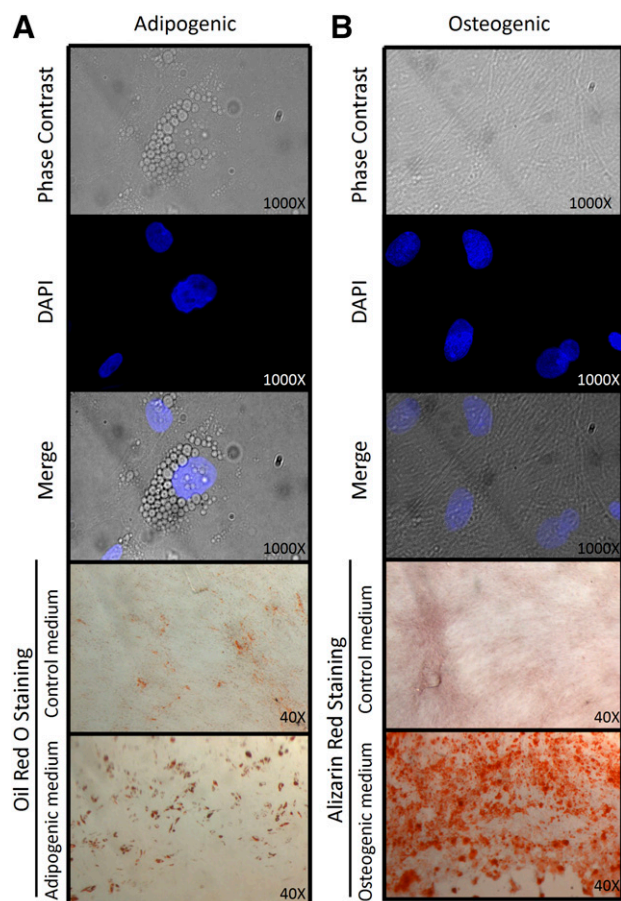

Figure 1. Lineage differentiation of adipose-derived mesenchymal stromal cells (ASCs). Cells (passage 7) were differentiated in adipogenic **(A)** and osteogenic **(B)** medium for 21 days. As a control, the cells were incubated for 21 days in ASC expansion medium. The production of lipids was assessed with Oil Red O staining **(A)**. The later stage of osteogenesis, characterized by calcium deposition, was assessed 3 weeks after induction via alizarin red-S staining **(B)**. Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

To establish the effect of TGF-β1 exposure on the ASC secretome, we analyzed an array of 80 cytokines/chemokines in the conditioned medium of ASCs either treated with TGF-β1 or untreated. We found that in both conditions, and based on relative protein abundance, ASCs secreted high quantities of interleukin-6 (IL-6), IL-8, C-C motif ligand 2, 7, and 11 (CCL2, CCL7, CCL11, respectively), tissue inhibitor of metalloproteinase-2 (TIMP-2), and angiogenin (Fig. 2A); intermediate levels of C-X-C motif 1, 2, and 3 (CXCL1, CXCL2, and CXCL3), together, CCL5, CXCL5, CXCL6, macrophage migration inhibitory factor, osteoprotegerin (OPG), and TIMP-1 (Fig. 2B); and low and very low levels of IL-1α, IL-5, IL-10, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), TNF-β, CCL8, CCL18, CXCL1, CXCL10, vascular endothelial growth factor, hepatocyte growth factor (HGF), placental growth factor (PIGF), leptin, fibroblast growth factor-7 (FGF-7), insulin growth factor binding protein-3 (IGFBP-3), leukemia inhibitory factor (LIF), oncostatin M (OSM), granulocyte/macrophage colony-stimulating factor (GM-CSF), and granulocyte-CSF (G-CSF) (Fig. 2C).

We also found that TGF-β1 downregulated the secretion of CCL7, CCL11 (Fig. 2A), CXCL6, OPG (Fig. 2B), and IL-5, IL-10, CCL8, CXCL1, CXCL10, HGF, leptin, FGF-7, GM-CSF, and G-CSF (Fig. 2C). In contrast, TGF-β1 stimulation upregulated PIGF,
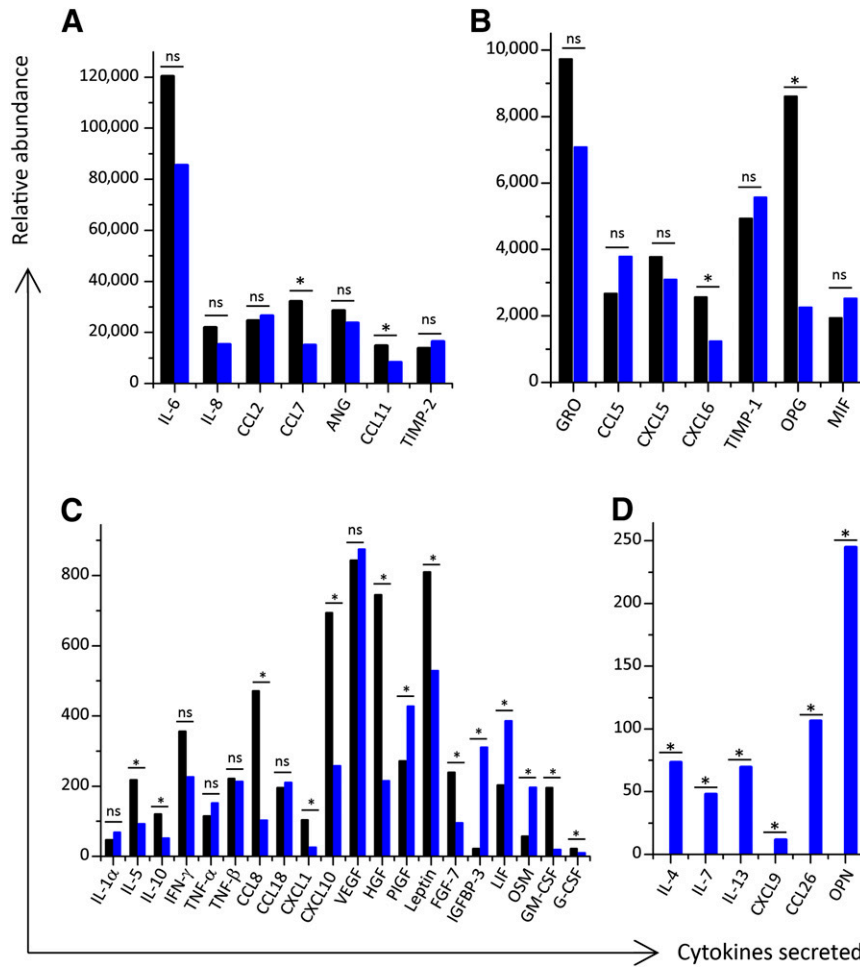
Figure 2. Semiquantitative secretion analysis of cytokines/chemokines in adipose-derived mesenchymal stromal cells (ASCs). Protein levels secreted by ASCs either treated with transforming growth factor-β1 (TGF-β1) (blue) or untreated (black) were analyzed by cytokine array. Cytokines/chemokines were grouped arbitrarily into four classes: high levels of secretion (>10,000 relative units) **(A)**, intermediate levels (1,000–10,000 relative units) **(B)**, low and very low levels (<1,000 relative units) **(C)**, and cytokines only detected in TGF-β1-treated cells **(D)**. Good data quality and adequate normalization were ensured using the mean signal intensity of positive control spots on the reference array. Any ≥1.5-fold increase in or ≤ 0.65-fold decrease in signal intensity for a single analyte between samples or groups was considered a measurable and significant difference in expression (asterisks), provided that both sets of signals were well above the background (mean background ± 3 SDs, accuracy ≈99%). Abbreviations: ANG, angiogenin; CCL, C-C motif ligand; CSF, colony-stimulating factor; CXCL, C-X-C motif; FGF, fibroblast growth factor; G, granulocyte; GM, granulocyte/macrophage; GRO, C-X-C motif 1, 2, and 3 together; HGF, hepatocyte growth factor; IFN, interferon; IGFBP, insulin growth factor binding protein; IL, interleukin; LIF, leukemia inhibitory factor; MIF, macrophage migration inhibitory factor; ns, nonsignificant; OPG, osteoprotegerin; OPN, osteopontin; OSM, oncostatin M; PIGF, placental growth factor; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

IGFBP-3, LIF, and OSM (Fig. 2C), and, IL-4, IL-7, IL-13, CXCL9, CCL26, and osteopontin (OPN) (Fig. 2D) were otherwise not detected in the conditioned medium of unstimulated cells. Finally, TGF-β1 stimulation of ASCs positively modulated the secretion of both TGF-β1 and TGF-β2 (Fig. 3A), which correlated with increased TβRIIA mRNA levels (Fig. 3B). In our conditions, we were unable to find detectable levels of an additional panel of 38 cytokines/chemokines (supplemental online data).

DISCUSSION

In the present study, we documented a panel of cytokines/chemokines secreted by ASCs and how TGF-β1 stimulation modifies the secretion of 26 of them in FBS-free conditions. Although our analysis showed a pattern of secretion of ASCs

similar to previous studies with MSCs from different sources [4–7], we still found some discrepancies. It is known that FBS contains numerous soluble factors suggested to be cross-reactive to human antibodies [4]. However, it cannot be ruled out that these bovine factors could also stimulate human cells to secrete cytokines that otherwise would not be secreted. In our study, to decrease this possible influence to a minimum, we performed the secretome analysis using cells starved for 16 hours and supplemented further with a low concentration of BSA for 3 days. Thus, the discrepancies found between us and others in the MSC secretome might be explained because these analyses were performed on MSC-conditioned medium supplemented with FBS [4–7]. Similar to our study, MSCs from different sources were also shown to secrete TGF-β1 and TGF-β2 but not TGF-β3 [7, 11–13].
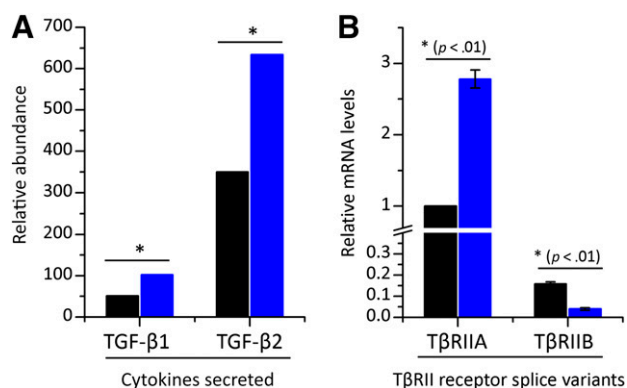
Figure 3. Effect of TGF-β1 stimulation on adipose-derived mesenchymal stromal cell (ASC) secretion of TGF-β family members. **(A):** TGF-β1 and TGF-β2 levels secreted by ASCs either treated with TGF-β1 (blue) or untreated (black) were analyzed by antibody array. Good data quality and adequate normalization were ensured using mean signal intensity of positive control spots on reference array. Any ≥1.5-fold increase or ≤0.65-fold decrease in signal intensity for a single analyte between samples or groups was considered a measurable, and significant difference in expression (asterisks), provided that both sets of signals were well above background (mean background ± 3 SDs, accuracy ≈99%). **(B):** Relative mRNA levels of TβRIIA and TβRIIB in ASCs stimulated (blue) and not stimulated (black) with TGF-β1 measured using reverse transcription-quantitative polymerase chain reaction. Data are displayed as the mean ± SE (bar) made in triplicates. Abbreviations: TβRIIA and TβRIIB, transforming growth factor-β receptor type II splice variants; TGF-β1, transforming growth factor-β1.

TGF-β1 auto-induction by TGF-β1 itself has been reported in normal and transformed cells [10]. In the same line of evidence, we have documented that in ASCs, TGF-β1 stimulation upregulates both TGF-β1 and TGF-β2 secretion. Moreover, IL-4 and IL-13, shown in the present study to be upregulated, were also previously reported to increase TGF-β production by bone marrow derived-MSCs [14], a fact demonstrated to be critical in suppressing the allergic responses in an in vivo model of asthma. In line with our results, both cytokines were also associated with increased production of CCL26, a potent chemoattractant of eosinophils, in bronchial epithelial cells [15]. However, in our study, we found decreased secretion levels of GM-CSF, which is required for the maturation and survival, as well as the augmentation of chemotaxis, degranulation, and cytokine production by eosinophils [16], and G-CSF, known to stimulate granulocyte production, maturation, and effector function [reviewed in 17]. Additionally, we have documented that IGFBP-3 secretion, which has been demonstrated to effectively reduce all symptoms of asthma [18], is upregulated in ASCs stimulated with TGF-β1.

MSCs are not innately immunosuppressive but acquire this capability after activation with inflammatory cytokines such as IFN-γ combined with TNF-α, IL-1β, or IL-1α [19]. Although TGF-β1 was reported to be responsible for MSC-mediated inhibition of T cells [20], in a recent study, TGF-β1 and -β2 have been shown to play a pivotal role in the reversion of the immunosuppressive effect exerted by activated MSCs, acting in an autocrine manner [21].

In the present study, we have shown that TGF-β1 induction of TGF-β1 and -β2 secretion does not reflect significant changes in the levels of classic proinflammatory cytokines such as INF-γ, TNF-α, and IL-1α. Instead, we found decreased levels of leptin, a hormone known to activate proinflammatory cells [22]. Secretion of OPN,

which induces T-cell chemotaxis and costimulates T-cell proliferation [23], and IL-7, a nonredundant cytokine for T cell maturation and differentiation [24], were stimulated after TGF-β1 treatment in our study. In addition, our results were coincident with decreased secretion of classic immunosuppressive cytokines such as IL-5, IL-10, and HGF and FGF-7 (also known as keratinocyte growth factor) [25]. In particular, MSCs treated with neutralizing antibodies against HGF or IL-10 have been demonstrated to partially reverse their immunosuppressive function on the proliferation of T cells [20, 26]. In contrast, the two functionally and structurally related cytokines LIF, known to be immunosuppressive [27], and OSM, shown to be anti-inflammatory without concordant immunosuppression in animal models of chronic inflammatory diseases (reviewed in [28]), were upregulated in our study.

It is known that activation of MSCs elicits very high levels of immunosuppressive factors and a burst of chemokine expression, including CXCR3 ligands (CXCL9, CXCL10, CXCL11) and CCR5 ligands (CCL3, CCL4, CCL5) (reviewed in [1]). In contrast, in the present study, we found that after TGF-β1 stimulation, most of the CCR5 ligands were either absent or unaffected and the CXCR3 ligands were downregulated, except for CCL9. Furthermore, other chemokines such as CCL7, CCL8, and CXCL6 were also downregulated by TGF-β1 in our study.

Another important cytokine normally secreted by MSCs is the anticlastogenic OPG, reported to be induced by TGF-β1 [29]. However, it has been found that OPG mRNA levels can be either decreased or increased, depending on the concentrations of TGF-β [30]. We document in the present study that TGF-β1 stimulation of ASCs largely downregulates OPG secretion. This observation has been confirmed by us, studying the conditioned medium of ASCs overexpressing TβRII-DN (Tania M. Rodriguez, Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús, Argentina, 2014, unpublished observation). With this dominant negative mutant of the type II receptor—a known inhibitor of the TGF-β signaling cascade—we observed that TGF-β1 stimulation suppressed TGF-β1 secretion but the OPG levels remained unaffected. Finally, we have shown that the potent angiogenic cytokine PIGF is upregulated by TGF-β1-stimulated ASCs. It has been hypothesized that PIGF functions in a concentration-dependent fashion, promoting osteogenesis at lower concentrations and angiogenesis/osteoclastogenesis at higher concentrations [31]. Our findings regarding the low levels of OPG and high PIGF secretion, along with cytokines with resorptive activity such as OPN [32], LIF, and OSM (reviewed in [33]), suggest a critical role for the interplay between TGF-β and ASCs in bone loss.

CONCLUSION

Our data show that in FBS-free conditions, ASCs secrete 36 of the 80 cytokines/chemokines analyzed and that TGF-β1 stimulation modulates the secretion of 18 cytokines and 8 chemokines. This modulation suggests that increased TGF-β1 and TGF-β2 secretion would actively participate to help ASCs to suppress allergic responses, stimulate bone resorption, and keep them mainly inactive for T-cell immunosuppression.

AUTHOR CONTRIBUTIONS

T.M.R.: collection and/or assembly of data, data analysis and interpretation, manuscript writing; A.S. and M.I.: provision of study materials or patients; M.J.P.: data analysis and interpretation; J.V.Z.: financial support; R.A.D.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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