Mesenchymal stem cells overexpressing interleukin-10 attenuate collagen-induced arthritis in mice


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Summary

Mesenchymal stem cells (MSCs) have the inherent ability to migrate to multiple organs and to exert immunosuppressive activity. The aim of this study was to investigate the anti-arthritisic effects of interleukin (IL)-10-transduced MSCs (IL-10-MSC) on the development of inflammatory arthritis. DBA/1 mice were immunized with type II collagen (CII) to induce inflammatory arthritis and then injected weekly three times with IL-10-MSCs 21 days after primary immunization. Control mice received vehicle or MSCs alone. Serum anti-CII antibody and T cell response to CII were determined. The results showed that cultured IL-10-MSCs were able to secrete high amounts of IL-10 in vitro. Injection of IL-10-MSCs decreased the severity of arthritis significantly. However, there was no difference in arthritis severity between mice treated with MSC and vehicle alone. Anti-CII antibody titres in the sera and T cell proliferative response to CII in lymph node cells were decreased significantly in mice treated with IL-10-MSCs compared with vehicle-treated mice. Serum IL-6 level was also decreased by the administration of IL-10-MSCs. In contrast, spleen cells of IL-10-MSC-treated mice produced higher amounts of IL-4 than those of control mice. Interestingly, although not as potent as IL-10-MSCs, injection of naive MSCs alone decreased serum levels of IL-6 and anti-CII antibody, while increasing IL-4 production from cultured splenic cells. Taken together, systemic administration of genetically modified MSCs overexpressing IL-10 inhibits experimental arthritis not only by suppressing autoimmune response to CII but also by regulating cytokine production, and thus would be a new strategy for treating rheumatoid arthritis.

Keywords: IL-10, mesenchymal stem cells, rheumatoid arthritis

Introduction

Mesenchymal stem cells (MSCs) are pluripotent progenitor cells of the adult marrow with the ability to differentiate into multiple lineages of the mesenchyme, such as chondrocytes, osteocytes, adipocytes and myocytes [1,2]. These primitive progenitors exist postnatally and exhibit stem cell characteristics, namely sparse population and extensive renewal potential [3]. It is relatively easy to isolate MSCs from bone marrow and expand them in vitro [4–6]. In addition, they can deliver efficiently exogenous genes and migrate to the damaged organ with the genes. By virtue of these advantages with their developmental plasticity, the perspective that the use of MSCs will be a promising tool for repairing damaged tissues as well as for gene therapy has been posited [7]. MSCs also have low immunogenicity and somewhat immunosuppressive properties. For these reasons, clinical studies have been tried to test whether the MSCs reduce graft-versus-host reactions in allogeneic transplantation [8,9]. Moreover, such a capacity to suppress T lymphocyte activities provides a rationale for applying these cells to the treatment of several autoimmune diseases [10], in which T cell hyperactivation contributes to pathogenesis such as rheumatoid arthritis (RA) and multiple sclerosis.

While the aetiopathogenesis of RA remains unclear, a variety of cytokines has been implicated in the development of this disease. In particular, cytokines secreted from macrophages or synoviocytes such as tumour necrosis factor (TNF)-α and interleukin (IL)-6 contribute to the induction of proinflammatory mediators as well as...
matrix metalloproteinase that could be responsible for joint destruction [11]. In contrast, IL-10 is a 35-kDa homodimeric cytokine that is produced by T helper type 2 (Th2) cells, activated monocytes/macrophages and certain regulatory T cells. IL-10 inhibits the production of Th1 and proinflammatory cytokines, including interferon (IFN)-γ, IL-2, IL-12 and TNF-α [11,12]. IL-10 is found at high levels in serum and synovial fluid of RA patients [13,14]. In an animal model of arthritis, IL-10-deficient mice showed an increase in disease severity [15]. Moreover, systemic treatment with IL-10 or adenovirus-mediated transfer of IL-10 is able to suppress the development of arthritis [16–20]. The suppression of IL-10 production by synovial cells is associated with increased levels of IL-1 and TNF-α [15,21], suggesting that IL-10 plays a protective role as an endogenous inhibitor in inflammatory arthritis [15,21].

The purpose of this study was to examine the therapeutic effect of MSCs and genetically engineered MSCs that express IL-10 on severity of murine collagen-induced arthritis (CIA). This study demonstrates that systemic administration of IL-10-transduced MSCs can delay the onset and reduce the clinical severity of CIA. In parallel, both serum levels of anti-CII (type II collagen) antibody and T cell response to CII in lymph node cells were reduced significantly in mice treated with both MSCs alone and MSCs overexpressing IL-10 (IL-10-MSCs), but the T cell responses were reduced significantly only by IL-10-MSCs, and not by MSCs alone. These results confirm the protective role of IL-10 in inflammatory arthritis, and provide a rationale for using MSC expressing IL-10 to treat RA.

Materials and methods

Mice

Male DBA/1 J mice, 6–10 weeks of age, were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The animals were kept in groups of five to six in polycarbonate cages and fed standard mouse chow and water.

Isolation and culture of MSCs

Bone marrow cells were collected by flushing the femurs and tibias with the medium, and then cultivated in 75 cm² tissue culture flasks at a concentration of 1 × 10⁶ cells/ml using complete Dulbecco’s modified Eagle’s medium (WelGENE Inc., Deagu, South Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS; WelGENE Inc.), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL, Gaithersburg, MA, USA). No cytokines were added at any stage of the experiments. The cultures were incubated at 37°C in a 5% CO₂ atmosphere. After 72 h, the non-adherent cells were removed. When the cells had reached 70% to 80% confluence, the adherent cells were trypsinized, harvested and expanded in larger flasks. A homogeneous cell population was obtained after culturing for 3–5 weeks. Established MSCs were subcultured until all cells become negative for hematopoietic marker CD45 [22].

Retroviral transduction

Retroviral transduction of IL-10 in the primary bone marrow-derived MSCs has been described previously [22,23]. Briefly, retroviral vector MIG (MSCV-IRES-GFP) and vector harbouring IL-10 (MIG-IL10) were co-transfected into 293T cells together with gag-pol, vesicular stomatitis virus-G and gibbon ape leukaemia virus envelope. Viral supernatants (5 × 10⁵–1 × 10⁶ infectious unit/ml) were then concentrated by ultracentrifugation, and used to infect MSCs at a multiplicity of infection of 10 for each transduction. The MSCs were then sorted for transduced [green fluorescent protein (GFP⁺)] cells by flow cytometric sorting and maintained in the culture medium containing 10% FBS. Expression of IL-10 in the transduced MSCs was confirmed by reverse transcription–polymerase chain reaction using primer sets specific to transgenic IL-10 as well as by measuring IL-10 levels secreted in the medium during 24 and 96 h of culture by enzyme-linked immunosorbent assay (ELISA).

Flow cytometric analysis of MSC

All the antibodies were purchased from PharMingen (San Diego, CA, USA). MSCs were incubated first with monoclonal antibody (mAb) 2G42 for 15 min at 4°C and then with the relevant fluorescein isothiocyanate- or phycoerythrin-conjugated mAb for 30 min at 4°C. Finally, the cells were washed twice with phosphate-buffered saline (PBS)/0.2% bovine serum albumin and fixed with PBS/1% paraformaldehyde. Flow cytometry was performed using a FACS Vantage SE cell sorter (Becton Dickinson). The identity of the MSCs was confirmed using the immunophenotype criteria, based on the expression of Sca-1⁻ and the absence of hematopoietic (with anti-CD45, -CD11c and -CD117 antibodies) or endothelial cell (with anti-flk-1 antibodies) markers. Moreover, both control-transduced or IL-10-transduced MSCs retained their osteogenic and adipogenic differentiation potential and exhibited a comparable phenotype of MSCs (CD45⁻, CD34⁻, flk-1⁻, CD31⁻ and Sca-1⁻), as described previously [23]. The proportion of CD45⁻ cells in the MSC preparations did not exceed 2% [22].

Determination of the effect of IL-10-transduced MSCs on CIA

Male DBA/1 mice were immunized with native bovine CII (Chondrex, Seattle, WA, USA) at 8–12 weeks of age, as described previously [24]. On days 21, 28 and 35 after primary immunization, mice were injected intravenously with IL-10-transfected MSCs diluted in Hank’s balanced salt solution.
solution containing 2% fetal calf serum (StemCell Technologies, Vancouver, Canada). Control mice received equal volumes of PBS or MSCs (1 × 10⁵) alone at the same time-points. Development of CIA was assessed every 2–3 days by an established macroscopic scoring system. Each joint was assessed on a 0–4 scale, as described previously [25]. The hind paw in which the booster immunization had been administered intradermally was excluded from the evaluation. Thus, the maximum possible arthritis score was 12.

Cytokine assay

The amounts of IL-10 and IL-4 released into the culture supernatants from either MSCs or splenic cells and the level of IL-6 in the sera were measured by ELISA.

Assay for immunoglobulin G antibodies to CII

Sera were collected from each group of mice on day 45 after the primary immunization, and stored at −20°C until assayed. The immunoglobulin G (IgG) anti-CII levels in the sera were determined by a commercially available ELISA kit (Chondrex). The optical density of the standard serum, which was diluted serially twofold, was expressed as 100, 50, 25, 12.5 and 6.25 arbitrary units respectively. The relationship of the optical density measured in the standard serum, diluted serially, and the arbitrary units showed good linear correlation in all determinations (γ = 0.98, data not shown). The IgG anti-CII concentrations in the sera, diluted 1:1000, are presented as relative values (arbitrary units) compared with the optical density of the standard sera.

Assessment of T cell proliferation

Lymph nodes and spleen were removed from each group of mice 45 days after primary immunization and washed in RPMI-1640. Tissues from four or five mice were pooled, minced into single-cell suspensions in RPMI-1640 and cultured in triplicate in 96-well flat-bottomed microtitre plates (Nunc, Roskilde, Denmark) with or without 40 μg/well of CII in total volume of 200 μl. Cells were incubated for 4 days at 37°C in 5% CO₂. During the last 6 h of incubation, cells were pulsed with 1 μCi of [³H]-thymidine and were harvested using an automatic harvester. Radioactivity was washed three times. The lymph node cells (5 × 10⁶) alone at the same time-points. Development of CIA was assessed every 2–3 days by an established macroscopic scoring system. Each joint was assessed on a 0–4 scale, as described previously [25]. The hind paw in which the booster immunization had been administered intradermally was excluded from the evaluation. Thus, the maximum possible arthritis score was 12.

Statistical analysis

Statistical comparisons were performed with Student’s t-test or Mann–Whitney U-test to compare non-parametric data for statistical significance. Percentage comparisons were performed using the χ² test. P-values less than 0.05 were considered significant.

Results

Generation of MSCs overproducing IL-10

We examined first whether the IL-10 gene, which was co-transfected with retrovirus expressing GFP, is transected efficiently in MSCs. Using flow cytometry analysis for GFP, we identified that almost all (> 95%) transduced MSCs were sorted as GFP⁺ cells [22], indicating that the IL-10 gene was introduced successfully into the cells. IL-10-transduced MSCs (IL-10-MSCs) preserved their characteristic phenotypes (CD45⁺, CD34⁺, CD11b⁺, CD106⁺, Sca-1⁻, CD80⁺, c-kit⁺, flk-1⁻, and CD90⁻) and were able to retain their multi-lineage differentiation potential, as described previously [22,23]. In fact, in order to obtain IL-10-MSCs expressing their unique phenotypes stably, only adherent cells had to be subcultured for at least eight passages. Subsequently, we tested the production of IL-10 in the culture supernatants of IL-10-MSCs using ELISA. The result showed that considerable amounts of IL-10 (22 679 ± 482 pg/ml per 4 × 10⁴ cells for 48 h) was produced from IL-10-MSCs, but rarely from naive MSCs without IL-10 gene (8.3 ± 9.5 pg/ml per 4 × 10⁴ cells for 48 h) (Fig. 1a). Moreover, the levels of IL-10 were increased in proportion to the number of IL-10-MSCs as well as over incubation time from 24 to 96 h. The IL-10 production by MSCs expressing the IL-10 gene was largely unaffected by the passage of subculture (Fig. 1b), indicating the high stability of MSCs for IL-10 production as a vehicle harbouring the IL-10 gene.

Effect of MSCs and IL-10-producing MSCs on the development and severity of CIA

To investigate the influence of MSCs and IL-10-MSCs on the onset and severity of arthritis, mice with CIA were divided into four groups; mice injected with PBS alone (n = 12), naive MSCs (n = 10), MSCs transfected with control MIG vector (n = 10) and MSCs transfected with MIG vector encoding IL-10 (IL-10-MSCs; n = 10). As shown in Fig. 2, IL-10-MSCs reduced the severity of arthritis significantly during the course of CIA. However, naive MSCs could delay arthritis onset while they failed to reduce severity of arthritis. In addition, there was no difference in the onset and severity of arthritis between mice treated with MSCs and mice with MIG vector control (data not shown), suggesting that the MIG vector did not contribute to arthritis suppression by IL-10-MSCs. Histological examination of the joints of the CIA mice also revealed a significant decrease in joint destruction, inflammatory cell infiltration and synovial hyperplasia in IL-10-MSCs-treated mice, but not in MSC-treated mice (Fig. 3a–e). These results, together with the in vitro data on
IL-10 production (Fig. 1), suggest that high levels of IL-10 secreted from IL-10-MSCs play a major role in suppression of arthritis, whereas MSCs themselves had little effect.

Inhibition of autoimmune responses to CII by MSC overexpressing IL-10

To investigate the effect of the IL-10-MSCs on the CII-specific immune responses, we measured the IgG antibodies to CII in the sera and the T cell proliferative responses to CII in the draining lymph node cells 6 weeks after primary immunization. As expected, titres of anti-CII antibody were significantly lower in mice treated with IL-10-MSC than PBS-treated mice, although they were also lowered in naive MSC-treated mice (Fig. 4a). In contrast, mice treated with IL-10-MSCs showed increased production of IL-4 from cultured splenic cells, an anti-inflammatory cytokine, compared with untreated control mice ($P < 0.01$) (Fig. 5b).

Interestingly, although not as potent as IL-10-MSCs, injection of naive MSCs alone into CIA mice decreased significantly serum levels of IL-6 and anti-CII antibody, while increasing IL-4 production from

Fig. 1. Production of interleukin (IL)-10 by mesenchymal stem cells (MSCs) after retroviral IL-10 transfection. The IL-10 levels in the supernatant were measured according to time–course (a) and passage of subculture (b) by enzyme-linked immunosorbent assay (ELISA). The results are representative of three independent experiments with similar results, and expressed as mean ± standard deviation. P10 = passage 10th, P11 = passage 11th.

IL-10 production (Fig. 1), suggest that high levels of IL-10 secreted from IL-10-MSCs play a major role in suppression of arthritis, whereas MSCs themselves had little effect.

Fig. 2. Anti-arthritic effect of systemically delivered mesenchymal stem cells/interleukin-10 (MSC/IL-10) in the collagen-induced arthritis (CIA) model. Mice were immunized with type II collagen (CII) on day 0. On day 14, mice received a booster of CII. Mice ($n = 10$, each group) then received intravenously $1 \times 10^6$ MSCs or IL-10-MSCs on days 21, 28 and 35 after primary immunization. Control mice ($n = 10$) received intravenous injections of an equal volume of phosphate-buffered saline at the same time-points. Development of CIA was assessed every 2–3 days in a blinded fashion, and a macroscopic scoring system was used as described in Methods. Results are expressed as the mean ± standard error of the mean of clinical scores and are representative of four independent experiments. Decrease in mean arthritis index in mice treated with IL-10-MSC (*$P < 0.05$; **$P < 0.01$ versus CIA mice).
cultured splenic cells (Figs 4 and 5). These results indicate that MSCs themselves, in essence, have an immune-suppressive potential, although they were not beneficial in curing RA. Furthermore, the ratio of IL-4/IFN-\(\gamma\) was increased markedly in mice injected with IL-10-MSCs (mean \(\pm\) standard deviation), 5·16 \(\pm\) 0·60; \(P < 0·01\) versus control or MSC-treated mice respectively), when compared with the control mice (0·94 \(\pm\) 0·25) and MSC-treated mice (2·95 \(\pm\) 0·46). Taken together, these data suggest that systemic administration of IL-10-MSCs inhibited a shift Th1 response as well as autoimmune responses to CII, and thereby suppressed severity of CIA in mice.

Discussion

In the present study, we assessed the anti-arthritic effect of MSCs and IL-10-transfected MSCs administered intravenously in CIA, a murine experimental model for RA. Because RA is a systemic inflammatory disease and MSCs may be capable of migrating into the damaged tissue, systemic delivery of MSCs is preferred to intra-articular administration. A recent study showed that systemic administration of MSCs leads to a significant prolongation of skin graft survival similar to the immunosuppressive agents currently being used clinically [26]. It is also well known that MSCs can inhibit T cell proliferation induced by allogeneic cells in vitro and mediate an immunosuppressive activity in vivo [27–29].
IL-4 by ELISA.

were immunized with CII, as described in Materials and methods. On Effect of IL-10-MSCs on IL-4 production in splenic cell culture. Mice

Data are the mean  

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TNF- 

immunomodulatory molecules secreted by naive MSCs.  

arthritis indices between 26 and 28 days may be due to the  

[35], although transiently significant reductions in mean  

results are consistent with a previous report where MSCs  

arthritogenic effects of IL-10-MSCs are due, at least in part,  

node cells and to increase IL-4 production in spleen cells  

be needed [35]. However, our observations that IL-10-MSCs  

In a first report for MSC in CIA, an immortalized cell line  

More recently, a study has demonstrated that MSC-mediated  

immunosuppression is shared by adult stromal cells such as  

fibroblasts, suggesting that stromal cells would also have  

important therapeutic implications as alternative tools for  

cellular therapy and immunoregulator [30].  

We have demonstrated here that genetically modified  

MSCs expressing IL-10 inhibited the onset and severity of  

arthritis in mice, suggesting that IL-10-MSCs may be useful  

for treating RA. It has been demonstrated that IL-10 is one  

of the most potent anti-inflammatory cytokines, and inhibits  

the progression of CIA [31–34]. Therefore, our data,  

together with earlier reports [31–34], suggest that the anti-  

arthritogenic effects of IL-10-MSCs are due, at least in part,  

to IL-10-mediated suppression of arthritis progression.  

Our results are consistent with a previous report where MSCs  

alone did not show any therapeutic benefit in the CIA model  

[35], although transiently significant reductions in mean  

arthritis indices between 26 and 28 days may be due to the  

immunomodulatory molecules secreted by naive MSCs.  

However, immunosuppressive characteristics of MSCs  

themselves were overwhelmed or even reversed by arthritic  

condition in which proinflammatory cytokines, such as  

TNF-α and IL-6, function dominantly [35].

Our work, showing arthritis suppression by IL-10-MSCs,  

however, contrasts sharply with a previous report by Djouad  

et al. [35]. A possible explanation for this discrepancy would  

be the difference in the amount of IL-10 administered. In  

this study, we injected the IL-10-transfected MSCs more  

frequently; shortly before the expected onset of CIA, and then  
twice more at interval of a week. Moreover, IL-10-MSCs used  
in our study were able to produce much more IL-10 than  
those used by Djouad et al. (22 ng/ml for 48 h per 4 ¥ 10^6  
cells versus 580 pg/ml for 24 h per 10^6 cells). In the case of  
IL-10 gene transfer with adenovirus in arthritic mice, a  
significant benefit was evident with 30 ng/ml of IL-10 in the  
sera, whereas 600 pg/ml of IL-10 had only a minimal effect  
[33]. Like other similar studies, IL-10 was not detectable in  
the sera following IL-10-MSC administration [35]. In  
particular, the systemic administration of recombinant IL-10  
in patients with RA showed toxic effects even if it had a  
tendency to exert clinical improvement [36]. Therefore, it seems  
likely that the abundant secretion of IL-10 from MSCs to  
reach a therapeutic range in local tissue levels is crucial to  
MSC-IL-10-induced suppression of arthritis. A recent study  
demonstrated that intravenously administered MSCs to CIA  
mice were recovered mainly from muscle and lung, although  
a more precise quantification for their biodistribution may  
be needed [35]. However, our observations that IL-10-MSCs  
were able to suppress T cell proliferation to CII in lymph  
node cells and to increase IL-4 production in spleen cells  
could be associated with sustaining the significant attenua-  
tion of CIA in both clinical and histological aspects,  
suggesting that these local immunological effects by IL-10  
in lymph nodes and spleen may play an important role in the  
therapeutic approach [33].  

In a first report for MSC in CIA, an immortalized cell line of  
MSC failed to mitigate CIA and was associated, rather,  
with accentuation of the Th1 response [35]. To the contrary,  
a subsequent report by Augello et al. using primary cultures  
of mouse MSC demonstrated that a single injection of cells  
(5 ¥ 10^6) suppressed CIA fully, inhibiting T cell response to  
CII as well as the expression of inflammatory cytokines [37].  

In this study, naive MSC (1 ¥ 10^6) alone decreased serum  
levels of IL-6 and anti-CII antibody, while increasing IL-4  
production from splenic cells, although they were not ben-  
eficial in ameliorating arthritis. This discrepancy may origin- 
ate from several factors, including the difference in kinds of  
MSCs (syngeneic versus allogeneic) and in the number of  
MSC used (5 ¥ 10^6 versus 1 ¥ 10^7). We believe that the thera-  
peutic effect on CIA may have been achieved by administrat- 
ing more amounts of MSC in our study.  

Interleukin-10 is a well-known inhibitor of T cell prolif- 
eration and proinflammatory cytokine production. In this  
study, administration of IL-10-MSC decreased serum levels  
of IL-6 and anti-CII antibody strongly, inhibiting T cell prolif- 
eration to CII both in vitro and ex vivo. Moreover, IL-10-  
MSCs increased IL-4 production of splenic cells, whereas  
they decreased levels of anti-CII IgG2a isotype (data not
shown). In our culture conditions for spleen cells in the presence of CII, we found that the production of IFN-\(\gamma\), TNF-\(\alpha\) and IL-17 by CII was not significantly different between mice injected with MSCs and IL-10-MSCs. In our culture conditions for spleen cells in the presence of CII, we found that the production of IFN-\(\gamma\), TNF-\(\alpha\) and IL-17 by CII was not significantly different between mice injected with MSCs and IL-10-MSCs. However, the ratio of IL-4/IFN-\(\gamma\) in mice injected with IL-10-MSCs was enhanced markedly by approximately fivefold compared with the control mice. These observations suggest that arthritis suppression by IL-10-MSC is associated with inhibition of T cell proliferation, probably Th1 cells, through the secretion of IL-10. Alternatively, the IL-10-induced changes in cytokine milieu, if occurring highly enough to overcome proinflammatory activity, would help MSCs return to their native ability for immunosuppression. If MSCs are unopposed by proinflammatory cytokines, they could play a predominant role in the relenting proliferative response of Th1 cells. Further study will be needed to clarify these issues.

In conclusion, we demonstrate first that systemic administration of genetically modified MSCs overexpressing IL-10 inhibited experimental arthritis not only by suppressing autoimmune response to CII, but also by regulating cytokine production. Considering that MSCs alone showed anti-inflammatory activity, albeit less potent than IL-10-MSC, the anti-arthritic effect of IL-10-MSC-treated mice seems to be caused by the cumulative action of both MSC itself and the secreted IL-10. These data provide a novel insight into the anti-inflammatory function of MSCs favoring tumor growth in allogeneic animals. Blood 2003; 102:3837–44.


References