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Mesenchymal Stem Cells Ameliorate Adriamycin Induced Proteinuric Nephropathy

Hee Gyung Kang, M.D., Ph.D.[*, †], So Yeon Park, M.D., Ph.D.[†]
Il Soo Ha, M.D., Ph.D.[*, §], Hae Il Cheong, M.D., Ph.D.[*, †, §]
and Yong Choi, M.D., Ph.D.[*, §]

Department of Pediatrics[*], Seoul National University Hospital
Research Center for Rare Diseases[†], Kidney Research Institute[§]
Seoul National University College of Medicine, and
Department of Pathology[†], Seoul National University Bundang Hospital

= Abstract =

Purpose : Glomerulonephropathy (GN) often manifests as proteinuria and progresses to chronic renal failure without specific therapy. Mesenchymal stem cell (MSC) has been tried as a therapeutic agent in experimental GN, and previous studies showed that administration of MSC concomitantly to the insult inducing GN or via intra-renal administration ameliorated proteinuria. The purpose of this study was to test the therapeutic potential of MSC administered via intravenous route at the time of clinically evident proteinuria.

Methods : MSCs were administered intravenously via tail vain into the mice with adriamycin (ADR) induced nephropathy (ADR-GN), two weeks after ADR injection when massive proteinuria was evident. To test the capacity of MSC modulate the cytokine production in the inflammatory milieu, the concentrations of IFN-γ and IL-10 were measured in the supernatant of in vitro mixed lymphocyte culture (MLC) with or without additional MSC.

Results : MSCs administered intravenously into the proteinuric mice with ADR-GN accelerated the recovery of this experimental GN with disappearance of proteinuria in two weeks when the saline treated (control) mice still showed significant proteinuria. The mice treated with MSC also had a tendency of better survival. Addition of MSC decreased IFN-γ and increased IL-10 in the supernatant of MLC.

Conclusion : This study showed that MSC had a therapeutic potential even when administered in a more clinically relevant setting into a proteinuric glomerulonephropathy model. Further study to verify the mechanism and long-term safety of this phenomenon is required. (J Korean Soc Pediatr Nephrol 2010;14:32-41)

Key Words : Glomerulonephropathy, Immune modulation, Mesenchymal stem cell, Proteinuria

Introduction

Glomerulonephropathy (GN) often manifests as proteinuria and progresses to chronic renal failure in many cases [1, 2]. The etiology and pathophysiology of idiopathic GN are yet to be

elucidated in most cases, so specific treatment leading to a cure is not readily available. Instead, non-specific immunosuppressive agents are frequently used, often with unsatisfying outcomes and untoward drug toxicity, rendering search for the non- or less-toxic remedy for the condition desirable. In this aspect, mesenchymal stem cell (MSC) can be a candidate for such a less-toxic therapeutic agent.

MSC is an undifferentiated, multipotent adult stem cell of mesodermal origin. It has the capacity to differentiate into various mesodermal cells such as cartilage, bone, adipose tissue and muscle cells. MSC is also known to have therapeutic potential; the cells migrate to the injured tissue such as infarcted myocardium and assist its healing [3, 4]. Another aspect of MSC with therapeutic potential is its non-specific immune modulating effect, possibly though immunosuppressive cytokine production or induction of immune-modulating cells [5-8]. The therapeutic potential of MSC has also been documented in kidney injuries; administration of MSC to experimental models of acute renal injury resulted in homing of these cells to the injured kidneys and amelioration of the clinical manifestations of renal injury, through differentiating into renal tubular epithelial cell and/or paracrine mechanism [9-11].

Regarding GN, recent studies using the anti-Thy1.1. model of GN demonstrated that intrarenal injection of MSC at the time of insult accelerated the glomerular healing [12, 13]. These studies showed the potential of MSC as a therapeutic agent for GN, at least at the acute stage. However, in addition to the concerns of its long-term safety, MSC needs to be tested in a more clinically relevant setting; what would happen if MSCs were administered intravenously, rather than via intra-renal or intra-cardiac route? What would happen if MSCs were administered to a host suffering from full-blown proteinuric nephropathy, rather than at the time of introduction of insult? In another study using Adriamycin (ADR) to induce nephrosis in rats [14], only when MSCs were given concomitantly to ADR, which is also a less feasible condition clinically, these cells showed protective effect. In the present study, we tested the therapeutic potential of MSC administered via intravenous route at the time of clinically evident proteinuria. For proteinuric GN induction, we used a well-known murine model of ADR induced proteinuric nephropathy (ADR-GN). ADR exerts a toxic effect on the kidney and induces inflammation, which in turn injure the glomeruli of the kidney [15-17].

Materials and Methods

1. Isolation and Purification of MSC

MSCs were isolated from bone marrow of 8 week-old male Balb/C mice by their tendency to adhere tightly to plastic culture dishes as described previously [18]. Their mesenchymal potential to differentiate toward osteocyte or adipocytes was verified by incubating the cells in differentiation-inducing media as described previously [19]. Differentiation to osteocyte was confirmed by staining calcium-rich hydroxyapatite in the extracellular matrix using Alizarin Red S (Sigma-Aldrich Korea, Yongin-city, Korea). Adipogenic differentiation was

confirmed by the presence of highly refractive intracellular lipid vacuoles stained with Oil Red O (Sigma-Aldrich Korea, Yongin-city, Korea) [19].

2. Murine Model of GN (ADR-GN)

Female Balb/C mice of 8 weeks of age were purchased from the Orient (Seoul, Korea) and used. Animals were cared for in accordance with "the Guiding Principles in the Care and Use of Animals" approved by the American Physiological Society. The study protocol was reviewed and approved by the Review Board of Seoul National University Hospital. After 24-hour urine was collected from the individual mouse using metabolic cages, GN was induced by injecting 11.5 mg/kg adriamycin (ADR [doxorubicin HCl, Ildong pharmaceutical Co., Seoul, Korea], diluted in sterile 0.9% saline solution) intravenously via the tail vein. The dose of ADR was chosen on the basis of previous preliminary experiments.

In 14 days, the mice were divided into two groups and received an intravenous injection via tail vein as follows: Saline group, saline (n= 13); MSC group, MSC (1×10^6 cells) derived from bone marrow of syngeneic mice (n=11). For In Vivo tracking of MSC, 2 additional mice (one ADR treated mouse and one control mouse) received MSCs labeled as previously described (20) with a tracking fluorochrome, 5-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probe, Inc. Portland, OR).

3. Monitoring the clinical findings of ADR-GN

Urine samples were collected at 0, 2, 4, 6 weeks after GN induction for urine protein/ creatinine ratio (uPr/Cr) measurement. At 6 weeks after GN induction, the mice were anesthetized with ether, blood samples for blood urea nitrogen (BUN), Cr, total protein, albumin determination were collected, and both kidneys were rapidly removed after perfusion with ice-cold phosphate-buffered saline using cardiac catheterization.

For the histological examination, tissues were fixed in 10% formalin and embedded in paraffin. Sections (4-μm) were stained with H&E staining and the degree of glomerulopathy was scored semi-quantitatively as described previously [21]. The degree of mesangial matrix expansion was scored by estimation of the percentage of each glomerulus occupied by mesangial matrix (1; 0-24%, 2; 25-49%, 3; 50-74%, 4; 75-100%). The mean value of the scores from 30 random glomeruli of a blind slide was used as the score of the slide.

For in vivo tracking of MSC, the kidney tissues harvested from CFSE-labeled MSC-injected mice were frozen, cut into 5-μm sections, fixed in 2% paraformaldehyde and examined with fluorescence microscope (Olympus VENOX-AHBT3) to detect the cells showing green fluorescence.

4. Effect of MSC on inflammation

To explore the effect of MSC on immunologi-

cal factors, which had been claimed to cause the glomerular injury in ADR−GN [15−17], the influence of MSC on cytokine production in the inflammatory milieu was analyzed in in vitro mixed lymphocyte culture (MLC) with or without additional MSC. This method was chosen to avoid additional chemicals that could potentially affect MSC other than the inflammatory milieu. Briefly, single cell suspensions were prepared from the spleens of C57BL/6 mice (MHC H−2b, allogeneic to Balb/c mice) and Balb/c mice (MHC H−2d) and cells from Balb/c mice were used as stimulator after irradiation so as not to proliferate. Cells from both strains were plated in 96−well flat−bottom plates and cultured. In the culture plate, C57BL/6 cells responded to the allogeneic stimulation of irradiated Balb/c cells and made the inflammatory milieu in the absence of additional chemical, as described elsewhere [22]. In some wells 1×10^5 MSC were added. In 72 hours, supernatant was collected from each well and the concentrations of IFN−γ and IL−10 in the supernatant were measured using Bio−Plex 200 (Bio−Rad, Richmond, CA).

5. Statistical analysis

The results are expressed as means± standard deviations. Statistical analysis of the data was performed using the Mann−Whitney U test for comparison between groups, or the Wilcoxon signed ranks test for comparison between two time points within a group, using SPSS for Windows version 10.0. Statistical significance was defined as $P<0.05$.

Results

### 1. MSCs have the potential to differentiate to osteocytes and adipocytes

The isolated MSCs used in the present study showed their potential to differentiate to two different mesenchymal tissue cells when incubated under appropriate culture conditions as shown in Fig. 1. Osteogenic condition induced MSCs to produce extracellular matrix with mineral deposition (Fig. 1B), and adipogenic condition induced the cells to accumulate lipid vacuoles (Fig. 1D).
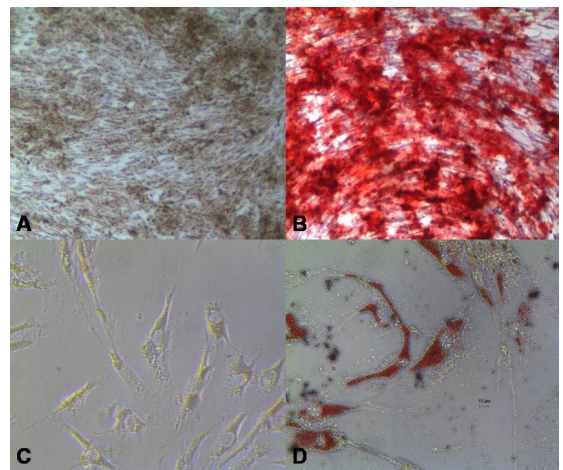
Fig. 1. MSCs have the potential to differentiate into osteocytes and adipocytes. MSC differentiated into producing extracellular matrix with mineral deposition (stained red, **B**) in osteogenic condition or to accumulate lipid vacuoles (stained red, **D**) in adipogenic condition. Undifferentiated MSC (**A** and **C**) did not show any staining by the same agents as B and D, respectively. Magnification; ×40 in A and B, ×400 in C and D.

2. MSCs ameliorate adriamycin induced proteinuria

In two weeks of injection, adriamycin induced severe proteinuria in Balb/c mice (uPr/Cr 75.3± 37.8 mg/mg, vs. baseline uPr/Cr 2.4±1.0 mg/ mg, Fig. 2). At this point, to study the effect of MSC on experimental GN, a group of the mice (MSC group, n=11) were injected with 1×10^6 syngeneic MSC while the others (Saline group, n=13) were injected with 0.9% saline. The amount of baseline and two weeks pro-teinuria were not different between the Saline group and the MSC group (uPr/Cr 2.7±1.0 vs. 2.0±0.8 mg/mg at baseline and 75.2±38.5 vs. 75.2±38.9 mg/mg at 2 weeks, respectively). At week 4, proteinuria decreased significantly in both the Saline (25.0±38.8 mg/mg [2 weeks vs. 4 weeks, $P=0.008$]) and MSC (6.5±5.8
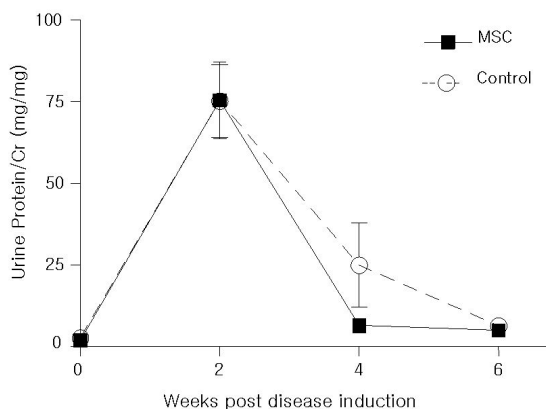
Fig. 2. MSCs ameliorate adriamycin induced pro-teinuria. In two weeks of injection, adriamycin induced severe proteinuria in Balb/c mice. Two weeks after the MSC or saline injection, the amount of proteinuria was still significantly higher than that of baseline in the Saline group ($P=0.011$), but the amount of proteinuria in the MSC group was not different from that of baseline ($P>0.05$).

mg/mg [$P=0.018$]) groups. While the amount of proteinuria was still significantly higher than that of baseline in the Saline group ($P=0.011$), the amount of proteinuria in the MSC group was not different from that of baseline ($P>0.05$). Two weeks later, both groups showed negligi-ble amount of proteinuria (6.4±5.2 in the Saline group and 5.1±3.6 mg/mg in the MSC group).
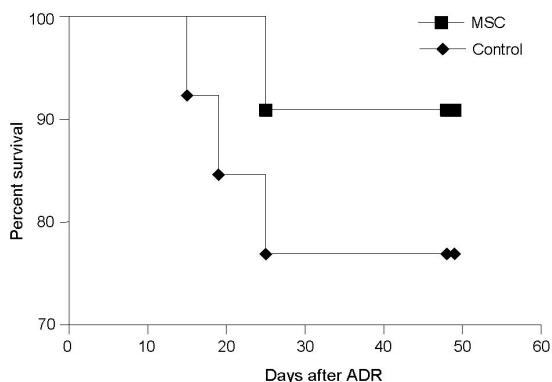
3. Administration of MSC favors survival in ADR-GN mice

Pathological evaluation at 6 weeks after the ADR injection showed a mesangial expansion score of 1.6±0.3 in the Saline group and 1.8± 0.6 in the MSC group without a statistical diffe-rence. Examination of the kidney tissues from CFSE-labeled MSC-injected mice with a fluo-rescence microscope did not reveal any cells with green fluorescence. At this point, other clinical findings in the Saline group and the MSC group of body weight (19.5±3.1 g vs. 21.1±1.3 g), serum albumin (4.2±1.0 vs. 4.0±0.7 mg/ dL), and serum creatinine (0.6±0.1 mg/dL vs. 0.6±0.2 mg/dL) were not different statistically. However, at 6 weeks after ADR-GN induction, the survival rate of the Saline group mice was 77% (n=10) while 90% of the mice in the MSC group (n=10) survived (Fig. 3). Although the Kaplan-Meier model of statistical analysis did not reveal statistically significant differences, such favorable survival of the MSC group was also observed in preliminary studies using dif-ferent dosages of ADR (data not shown).

4. MSCs modulate cytokine production in the inflammatory milieu

In ADR-GN, the glomerular injury by ADR is thought to be caused by immune factors as well as direct toxic effect of the drug on the glomerular cells [15-17]. Since in the present study MSC was administered at the later stage of ADR-GN when proteinuria was evident and the toxicity of ADR was not expected to linger,

we sought to explore the effect of MSC on the immunological factors using MLC, an in vitro model of inflammation, which does not require additional chemicals to induce immunological activation. When MSC was added to MLC, the cytokine concentration in the supernatant was affected by MSC (Fig. 4A). While IFN-γ, the well-known pro-inflammatory cytokine, was decreased, IL-10, the well-known anti-inflammatory cytokine, was increased in the supernatant of MLC when MSC was added to the culture (Fig. 4B).

Discussion

The purpose of this study was to test the therapeutic potential of MSC that was administered in a more clinically relevant setting of intravenous infusion via intravenous route at the time of clinically evident proteinuria. Here, MSC accelerated the decrease of proteinuria and favored survival of murine ADR-GN model. While there are several reports documenting MSC ameliorating acute renal injury and glomerulopathy, those studies investigated the

Fig. 3. Administration of MSC favors survival in ADR-GN mice. At 6 weeks after ADR-GN induction, the survival rate of the Saline group was 77% (n=10) while 90% of the mice in the MSC group (n=10) survived.
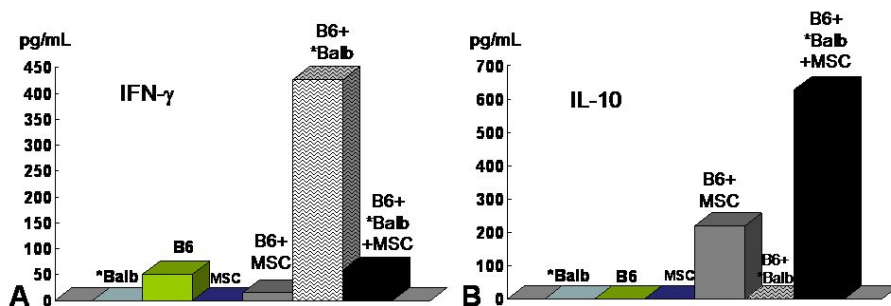
Fig. 4. MSCs modulate cytokine production in the inflammatory milieu. When MSC was added to MLC, IFN-γ, the well-known pro-inflammatory cytokine, was decreased **(A)** and IL-10, the well-known anti-inflammatory cytokine, was increased in the supernatant of MLC when MSC was added to the culture **(B)**. *indicating irradiated cells.

effect of MSC by administering MSC at the time of injury and via routes that are difficult to access clinically, such as through the aorta or intrarenal injection [12, 13]. Here, we chose to administer MSC in the setting that was clinically more feasible, and still MSC could ameliorate the nephropathy and favor the survival of the proteinuric hosts.

The limitation of this study is that the pathological evidence of the effect of MSC was not demonstrated. Since ADR-GN was self-limited or lethal despite the dose of ADR being chosen carefully on the basis of previous preliminary experiments using numerous mice, the comparison of pathologic findings between the mice surviving by the time of sacrifice (6 weeks after disease induction) failed to show any statistically significant difference. Another pitfall of this study is that the attempt to track MSC failed to demonstrate MSC in the kidney 4 weeks after administration of these cells; therefore, we are not sure if the administered MSC reached the injured kidney or not. It is well known that most of the MSC administered via intravenous route are captured at the pulmonary vascular bed soon after administration [23], and in previous studies an intra-aortic or intra-renal route of MSC administration was chosen to bypass the lung; On the other hand, there are studies clearly showing that the captured MSC is released from the pulmonary vascular bed and redistributed to peripheral organs including the kidney [24, 25]. Accordingly, we speculate that the intravenously introduced MSC reached the kidney and accelerated the recovery of ADR-GN.

Then, by which mechanism did MSC exert such an effect on the proteinuric nephropathy? Among the known therapeutic potentials of MSC, the early effect of MSC on acute renal injury is considered to be the paracrine or autocrine effect of MSC rather than the replacement of damaged cellular component of the kidney by differentiating into renal cellular components [10, 11, 26]. By the same token, we speculate that non-specific immune modulating effect of MSC might have worked in this model, through modulating cytokine production in the inflammatory milieu as shown previously [5]. Although the milieu 2 weeks after ADR injection, when the toxicity of ADR had already disappeared, was impossible to reproduce in vitro, we could verify that the MSC we used in this study had such an immune modulating effect (Fig. 4). Another possibility is that MSC secreted some factors accelerating the recovery of podocytes or other components of the kidney as shown previously [14]. Or, considering the favorable survival in the MSC group, MSC may have beneficial effect on the cardiovascular - hemodynamic system, manifesting less proteinuria in ADR-GN. Further study of pathologic evaluation at different time points might reveal better explanation, especially since a previous study did not show the potential of MSC to modify the clinical parameters in ADR model of rats when administered at different scheme [14].

Another important issue regarding MSC as a therapeutic agent is its safety concern. Since MSC has multipotency and the capacity of self renewal similar to that of malignant cells, safety concern has constantly been addressed. True to the concern, in previous study by Kunter et

al. some of the intra-renally introduced MSC into anti-Thy1.1. GN differentiated into intra-glomerular adipocytes 60 days later [13]. On the other hand, such a mal-differentiation was not demonstrated until 24 days later when these cells were injected via tail vein [25]. Perhaps intravenous MSC administration allowed recruitment of only desirable (number of) cells to the damaged tissue without redundant cells. Nevertheless, further study is required to confirm the long-term safety of MSC. Other questions yet to be answered are the efficacy MSC over pre-existing non-specific agents such as glucocorticosteroid especially in its capacity to suppress the progression of renal damage and the optimal form of MSC to be used.

In summary, this study showed that intravenous administration of MSC ameliorated the proteinuria of ADR-GN, a model of proteinuric nephropathy, and favored survival of the hosts. Further study to verify the mechanism and long-term safety of this phenomenon is required.

요 약

Adriamycin 유발 신병증에서 중간엽 줄기세포의 완화 효과

서울대학교 의과대학 소아과학교실[*],
희귀질환진단치료기술연구개발사업단[†]
서울대학교 의과대학 병리학교실[‡], 신장연구소[§],

강희경[*, †] · 박소연[†] · 하일수[*, §] · 정해일[*, †, §] · 최용[*, §]

목 적: 사구체신염은 흔히 단백뇨를 보이며 특이 치료법이 없고, 만성 신부전으로 발전하는 경우가 많다. 몇몇 연구에서 중간엽 줄기세포(Mesenchymal stem cell, MSC)를 실험적 사구체신염에 투여하여 단백뇨가 호전된 것을 보고한 바 있으나, 이는 신염을 일으키는 약제와 중간엽 줄기세포를 함께 투여하거나 신장에 직접 투여한 것이었다. 본 연구에서는 실험적 신병증에서 단백뇨가 발현된 시점에서 정주 요법으로 MSC를 투여함으로써 MSC의 임상적인 적용 가능성을 탐색하였다.

방 법: 실험용 생쥐에 Adriamycin을 투여하여 신병증(ADR-GN)을 유발한 후, 2주 후에 대량의 단백뇨를 확인하고 MSC를 생쥐 꼬리의 정맥에 주사하였다. MSC에 의한 질병 완화의 기전을 확인하기 위한 in vitro 실험으로 mixed lymphocyte culture (MLC)에 MSC를 투여하였을 때의 염증 관련 cytokine인 IFN-γ and IL-10의 변화를 측정하였다.

결 과: 실험용 생쥐에 ADR-GN를 유발하고 단백뇨가 보일 때 MSC를 정주한 군에서는 단백뇨의 소실이 더 먼저 관찰되었다. 또한 MSC를 투여받은 군에서의 생존률이 더 나은 경향이 관찰되었다. MLC에 MSC를 투여하였을 때, 염증을 유발하는 cytokine인 IFN-γ는 감소하고 염증을 억제하는 cytokine인 IL-10는 증가하였다.

결 론: 이 연구는 이전의 보고들에서 관찰되었던 사구체신염에서의 MSC의 질병완화 효과가 좀더 임상적으로 적용 가능한 방법으로 투여된 경우, 즉 단백뇨가 있을 때 정주 요법으로 투여한 경우에도 관찰됨을 확인하였다. 이러한 효과의 기전과 임상적용에 요구되는 안전성 등에 대한 확인을 위해서는 추가 연구가 필요하겠다.

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