Short paper

Allogeneic mesenchymal stem cell infusion for the stabilization of focal segmental glomerulosclerosis

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Article history:
Received 3 June 2013
Received in revised form 20 August 2013
Accepted 11 September 2013

Keywords:
Mesenchymal stem cells
Inflammatory factors
Focal segmental glomerulosclerosis

1. Introduction

Focal segmental glomerulosclerosis (FSGS) is the most frequent acquired renal condition resulting in end stage kidney disease (ESKD) in children [1]. In FSGS a common glomerular lesion mediated by diverse insults directed to or inherent within the podocyte leads to effacement of the podocyte foot processes. Consequently, there is a loss of integrity of the glomerular filtration barrier, which regulates permselectivity, that causes in turn nephrotic proteinuria [2]. FSGS is responsible for 5–20% of all cases of ESKD in the USA and is second only to urogenital and kidney malformations as a cause of ESKD in children [3]. It is assumed that approximately 80% of FSGS are primary (idiopathic). The rate of recurrence is higher in children compared with adults and in patients submitted to a subsequent kidney transplant [4] and its incidence is rising worldwide [5]. Furthermore, after kidney transplantation, approximately 30–40% of patients with FSGS develop recurrent FSGS [6,7]. The major goals of FSGS therapy are to achieve complete remission of proteinuria and to preserve kidney function. However, there are no therapeutic regimens that induce remission in all cases. Most agents used to treat FSGS are immunomodulators since most cases of idiopathic FSGS are thought to be part of the immune-mediated minimal change disease/FSGS disease spectrum. Corticosteroids are the mainstay of treatment for
idiopathic nephrotic syndrome; however, only 30% of children with FSGS histology will achieve remission with steroids [8]. The rationale for the use of corticosteroids in FSGS relies on the suppression of a T-lymphocyte-mediated response and/or on the effect on podocytes and their cytoskeleton. The use of Rituximab, a chimeric monoclonal antibody that inhibits CD20-mediated B lymphocyte proliferation and differentiation, was supported by the first evidence of the proteinuria normalization in a child affected by FSGS after kidney transplantation who received this drug, to treat a large cell B lymphoma [9]. Definitively, FSGS remains a severe disease unsuccessfully treated with traditional pharmacological approaches and therefore there is a huge need of alternative therapies for it [10]. Recently, human allogeneic bone marrow-derived (BM) mesenchymal stem cells (MSC) have been proven to be safe and effective in a wide range of immunemediated diseases [11–14].

Herein we report our experience with an innovative use of MSC in a pediatric recipient of kidney transplantation with a form of FSGS not responding to any conventional and unconventional treatments.

2. Subjects and methods

The ethics committee approved the treatment. The patient received the cell infusions after informed consent expressed by his parents.

A 13-year-old boy presented with immediate recurrence of focal FSGS after renal transplantation (Tx).

The patient was a firstborn with silent family history, except for one cousin of third degree with ESKD of unknown origin. He was in good health until he was 10-years-old, when he developed a nephrotic syndrome (edema, serum albumin <2.5 g/dL and proteinuria/creatinuria ratio uPr/uCr from 4 to 35). The patient was treated with prednisone (60 mg/sqm/mean per day) for 8 weeks, then with 3 pulses of methylprednisolone (500 mg/dose), with no effect on proteinuria.

All the secondary causes of nephrotic syndromes due to infections were excluded and therefore the patient was classified as suffering from a steroid-resistant nephrotic syndrome.

Thus, a renal biopsy was performed that showed a primary FSGS, suggestive for a classic variant [15]. Moreover, we performed an in vitro functional assay (P Alb) to evaluate the changes in permeability incubating the isolated glomeruli with the patient serum [16]. The test was positive with an expected risk of FSGS recurrence five times greater than patients with P Alb in normal values [17].

The genetic analyses for mutations in nephrin, podocin and WT1 were negative. He was treated with cyclosporine A (4 mg/kg/day) for 8 weeks with plasmatic levels ranging from 70 to 240 ng/mL (therapeutic range is 80–120 ng/mL) and then with tacrolimus (0.1 mg/kg/day) for twelve weeks, but no response was observed.

In consideration of worsening in renal function and persistence of nephrotic proteinuria despite of the immunosuppressive therapy, the biopsy was repeated and confirmed FSGS with a more evident tubular atrophy and interstitial fibrosis possibly secondary to calcineurin inhibitor toxicity.

Therefore, the immunosuppressive therapy was discontinued. After 9 months, he progressed to renal failure with a need for dialysis.

Eighteen months after starting hemodialysis, he received a kidney transplant from a cadaver adult donor with one match for HLA typing of patient, transplanted kidney, MSC I, MSC II and MSC III.

<table>
<thead>
<tr>
<th>Source</th>
<th>Locus A</th>
<th>Locus B</th>
<th>Locus DR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>3,68</td>
<td>35,49</td>
<td>11,15</td>
</tr>
<tr>
<td>Transplanted kidney</td>
<td>3,32</td>
<td>7,35</td>
<td>3,7</td>
</tr>
<tr>
<td>MSC I</td>
<td>1,2</td>
<td>7,8</td>
<td>3,1</td>
</tr>
<tr>
<td>MSC II</td>
<td>26,66</td>
<td>18,41</td>
<td>11,13</td>
</tr>
<tr>
<td>MSC III</td>
<td>3,24</td>
<td>7,51</td>
<td>4,12</td>
</tr>
</tbody>
</table>

TAC was started orally 12 h at a dose of 0.3 mg/kg with the aim of reaching target TAC blood concentrations between 10 and 20 ng/mL for the first month, then 5–10 ng/mL. MMF was administered orally at a dose 600 mg/sqm/b.i.d. Steroids (methylprednisolone) were given intravenously during the first 3 post-transplant days, and then orally as prednisone 1 mg/kg, which was gradually tapered by 0.25 mg/kg every two weeks until post-transplant day 90, followed by a dose of 0.1 mg/kg/day.

Two days after transplantation, he presented immediate recurrence of FSGS and a graft biopsy confirmed this diagnosis.

Indeed, PLF with replacement of 150% of 4% albumin solution was begun on POD 6, initially on a daily schedule. The frequency of PLF was progressively reduced basing on uPr/uCr values, in order to keep the ratio uPr/uCr below 5. The initial frequency was 4 times a week, then twice a week until it was delayed weekly (Fig. 1). To induce remission of FSGS, six weeks after Tx, 2 doses of intravenous Rituximab were administered (375 mg per sqm of body-surface area), but without any clinical improvement. In fact, PLF was stopped after each Rituximab administration only for 4 and 8 weeks, respectively. Moreover, after 5 months from the last dose, despite a suppression in the number of CD20 + CD19 + cells, no effect on uPr/uCr ratio was observed and the patient needed a weekly PLF in order to achieve a partial control of proteinuria (uPr/uCr < 5).

Seven, 10 and 14 months after Tx and 5 months after Rituximab administration, the patient received allogeneic BM MSC infusions. MSC were administered in 6 doses, divided in three cycles of 2 infusions (1 × 10⁸ cells/kg/dose) according to the dose most commonly used for GvHtreatment [16]. After the first MSC cycle, he did not need any PLF for 50 days. Because of a slow rising of uPr/uCr, 3 and 7 months after the first MSC infusion the patient was treated with a second and a third MSC dose.

The last PLF session (n = 46) was administered just before the second MSC cycle. A kidney biopsy was performed one year after cell infusions. Up to now, with a follow up of 22 months from the last MSC administration, the patient is doing well, the uPr/uCr ratio is maintained and no adverse events have been registered neither during infusion nor in the following period.

2.1. Cell production: MSC isolation and culture under good manufacturing practices—GMP conditions

Cell preparation was done at the Laboratorio di Terapia Cellulare “Stefano Verri”, authorized by Agenzia Italiana del Farmaco (AIFA) for the production of cellular-based medicinal products.

MSC were isolated from unrelated random BM donors, after having obtained written informed consent from the donors. Clinical-grade MSC were generated under GMP conditions, as recently described [19,20]. Operating conditions were previously reported [21]. Briefly, cells were manipulated in Grade A safety cabinet and surrounding Grade B clean room according to Eu-GMP rules.

Sterility and mycoplasma tests were performed according to European pharmacopeia (EP) guidelines (chapters 2.6.27 and 2.6.7).
respectively), endotoxin test was performed by limulus amebocyte lysate (LAL) method, according to EP (2.6.14) by using the Pyrogent®/C210 Plus (Lonza, Basel, Switzerland).

2.2. MSC viability and immunophenotyping

The MSC phenotype was analyzed by standard flow cytometry procedure (FACScalibur, Becton Dickinson, BD, San Jose, CA, USA) as previously described [19]. Release MSC phenotype was high expression (>70%) of CD73, CD90, CD105, HLA-ABC and lack (<10%) of CD33, CD34, CD14, CD45. MSC viability was evaluated either by Trypan blue dye exclusion or by propidium iodide staining as assessed by flow cytometry, and release MSC viability was higher than ≥80%.

2.3. Soft agar assay

Final MSC were plated at a density of 1000 cells/35-mm Petri dish (BD Falcon) in hematopoietic stem cell—colony forming units (HSC—CFU) basic methylcellulose media (code: 130-091-275, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). After 14-day incubation, colony-forming unit cells (CFU-C) were scored and counted under an inverted microscope. A positive control consisted of the Ewing’s sarcoma PDE-02 cell line.

2.4. MSC infusion

For all the infusions, cryopreserved allogeneic BMMSC (chosen without any regard to HLA-match, 1 × 10^6 cells/kg) were used after thawing at 37 °C and diluted in 90 mL of saline. MSC viability was tested before the cell infusions as previously reported. The cells were infused intravenously in 10 min using a standard 18 Gauge catheter.

2.5. Clinical and laboratory evaluation of renal function

Before and after MSC administration, the patient underwent an intensive urine and blood test monitoring program.

Samples for proteinuria were collected every other day. A clinical evaluation and a complete blood testing, including renal function, were performed every 2 weeks.

2.6. Short tandem repeat assays and donor-specific anti-HLA antibody screening

Allele peaks detected in the kidney biopsy specimens were compared with BMMSC of the three donors to determine whether the amplified alleles were attributable to DNA from the recipient, donors, or both. The method used was based on the amplification of nine short tandem repeat loci (D3S1358, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820) and a segment of an X—Y homologous gene.

Donor-specific anti-HLA antibody screening was performed by Luminex technology.

2.7. Proteome array

Blood samples of the patient were collected at different stages before, during and after MSC infusions and then centrifuged at 900 × g to collect sera.

The proteome profile was analyzed in the following serum samples: pre-BM-MSC infusion I (T0), 12 h post-infusion I (T12h-I), 12 h post-infusion II (T12h-II), 4 (T4m) and 5 months (T5m) after the first infusion, 12 h post-infusion III (T12h-III), and one year after the first infusion (T1y).

The dosage of biomarkers in serum samples was performed using Rules-Based Medicine’s multiplexed immunoassay service (Myriad RBM, Austin, TX) based on Luminex xMulti-Analyte profiling (MAP) platform. The most relevant markers of renal damage were quantified using the human KidneyMAP v1.0 multiplex immunoassay service (list of markers: http://www.myriadrbm.com/products-services/humanmap-services/human-kidneymap/).

In addition, a panel of 182 protein biomarkers comprising cytokines, inflammatory markers, growth factors and tissue factors in serum samples was investigated with the DiscoveryMAP™ v1.0
3.3. Biopsy and STR assay

Clinical edema was detected. Table 1 shows HLA typing of patient, transplanted kidney, MSC I, MSC II and MSC III. The screening for donor-specific anti-HLA antibodies was negative. DNA of both recipient and kidney donor was found 30% and 70%, respectively. No DNA from any BMMSC lines was detected by the ratio between pre-infusion value (pre-BM-MSC infusion I (T0)), after BM-MSC infusion III (T-III), one year after the first infusion (T1y) and two years after the first infusion (T2y).

The dosage of suPAR was performed using ELISA assay (Viro-Gates A/S, Birkered, Denmark) accordingly to the manufacturer's instructions.

3. Results

3.1. Cell production and immunophenotyping

All three BMMSC batches were compliant with defined specifications and successfully released (Table 2). The viability after thawing and before infusion was 92 ± 3%.

3.2. Patient follow-up

After a follow up of 22 months the patient had a normal and stable renal function (serum creatinine 0.96 mg/dl, GFR 94 mL/min/1.73m²). The uPr/uCr ratio mean values were below the value of 5 (see Fig. 1) with a mild hypoalbuminemia not associated with clinical edema.

3.3. Biopsy and STR assay

A renal biopsy revealed FSGS with mild mesangial proliferation, deposition of IgM and severe podocyte foot process effacement at the electronic microscope.

One year after cell infusions, the kidney biopsy still revealed the presence of FSGS. DNA of both recipient and kidney donor was found 30% and 70%, respectively. No DNA from any BMMSC lines was detected. Table 1 shows HLA typing of patient, transplanted kidney, MSC I, MSC II and MSC III. The screening for donor-specific anti-HLA antibodies was negative.

3.4. Proteome array

For each biomarker, a comparison at different steps of treatment was done by the ratio between pre-infusion value (pre-BM-MSC infusion I) and each single post-infusion value.

In order to select only factors that significantly changed during treatment, a cut-off obtained from ratios ≥3 fold was applied. Table 3 reports the biomarkers that significantly changed during MSC treatment: amphiregulin, brain-derived neurotrophic factor (BDNF), CD40 ligand, epidermal growth factor (EGF), extracellular newly identified receptor for advanced glycation end products binding protein (EN-RAGE), eotaxin-3, heparin binding-EGF (HB-EGF), interleukin-16 (IL-16), macrophage migration inhibitory factor (MIF), myeloperoxidase (MPO), N-terminal prohormone of brain natriuretic peptide (NT-proBNP), plasminogen activator inhibitor-1 (PAI-1), platelet derived growth factor-BB (PDGF-BB), superoxide dismutase-1 (SOD-1), transforming growth factor-alpha (TGF-alpha), thrombospindin-1. The control levels obtained from healthy donors of these markers in serum are lower than values present in our patient, a list of some factors is reported in literature: NT-proBNP (37.79 ± 18.77 pg/mL) [22], IL-16 (42.38 pg/mL) [23], EGF (2.4 ± 0.5 pg/mL) [24], eotaxin-3 (34.1 ± 15.3 pg/mL) [25], TGF-alpha and amphiregulin (6.0 ± 4.2 and 19.6 ± 17.4 pg/mL, respectively) [26].

3.5. suPAR

Serum levels of suPAR were always above the cutoff limit of 3000 pg/mL and showed only a minimal decrease after the third MSC infusion and successively remaining stable during the all follow-up (see Fig. 2).

4. Discussion

FSGS remains an important cause of renal failure worldwide. Advances in molecular genetics and cell biology significantly contributed to the understanding of podocyte biology and of the possible role played by podocyte dysfunction in the pathogenesis of FSGS. However, therapeutic advances have not matched this increase in knowledge. Still the majority of patients showing a nephrotic range proteinuria secondary to FSGS relapse, including the patient presented in this case report, lost renal function after transplantation. Currently, PLF is the most beneficial treatment early in the course of recurrence, leading to remission after 8 to 12 treatments [6]. In our patient a series of 46 consequent plasmaphereses were unable to keep proteinuria below acceptable values and if the time between two plasmaphereses was delayed, a disease exacerbation was observed (Fig. 1) with a consequent negative impact on an already poor quality of life of a chronic diseased teenager patient. In this context, an innovative MSC treatment has been proposed with the aim to modulate the microenvironment reducing inflammation thus possibly bringing direct benefits to glomerular functionality. First of all, allogeneic MSC infusion was
The proteome profile was analyzed in the following serum samples: pre-BM-MSC infusion I (T0), 12 h post-infusion I (T12h-I), 12 h post-infusion II (T12h-II), 4 (T4m) and 5 months (T5m) after the first infusion, 12 h post-infusion III (T12h-III), and one year after the first infusion (T1y). BDNF = brain-derived neurotrophic factor; EGF = epidermal growth factor; EN-RAGE = extracellular newly identified receptor for advanced glication end products binding protein; HB-EGF = heparin-binding-EGF; IL-16 = interleukin-16; MIF = macrophage migration inhibitory factor; NT-proBNP = N-terminal prohormone of brain natriuretic peptide; PAI-1 = plasminogen activator inhibitor-1; PDGF-BB = platelet derived growth factor-BB; SOD-1 = superoxide dismutase-1; TGF-alpha = transforming growth factor-alpha. Lowa values reflect samples not measurable on the standard curve. NDb = not detected.

Table 3
Biomarkers during MSC treatment.

<table>
<thead>
<tr>
<th>Biomarkers (ng/mL)</th>
<th>T0</th>
<th>T12h-I</th>
<th>T12h-II</th>
<th>T4m</th>
<th>T5m</th>
<th>T12h-III</th>
<th>T1y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphiregulin</td>
<td>1.61</td>
<td>0.859</td>
<td>1.56</td>
<td>0.964</td>
<td>0.176</td>
<td>Lowª</td>
<td>Lowª</td>
</tr>
<tr>
<td>BDNF</td>
<td>5.34</td>
<td>2.12</td>
<td>3.79</td>
<td>2.41</td>
<td>0.89</td>
<td>2.9</td>
<td>1.1</td>
</tr>
<tr>
<td>CD40 ligand</td>
<td>2.27</td>
<td>0.4</td>
<td>0.97</td>
<td>0.21</td>
<td>0.21</td>
<td>0.3</td>
<td>NDª</td>
</tr>
<tr>
<td>EGF</td>
<td>0.105</td>
<td>0.018</td>
<td>0.034</td>
<td>0.008</td>
<td>0.034</td>
<td>0.03</td>
<td>NDª</td>
</tr>
<tr>
<td>EN-RAGE</td>
<td>8.99</td>
<td>2.41</td>
<td>3.91</td>
<td>1.28</td>
<td>0.53</td>
<td>0.4</td>
<td>NDª</td>
</tr>
<tr>
<td>Eotaxin-3</td>
<td>0.459</td>
<td>0.134</td>
<td>Lowª (&lt;0.070)</td>
<td>Lowª (&lt;0.070)</td>
<td>Lowª (&lt;0.070)</td>
<td>Lowª (&lt;0.070)</td>
<td>Lowª (&lt;0.070)</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>0.379</td>
<td>0.207</td>
<td>0.334</td>
<td>0.242</td>
<td>0.025</td>
<td>0.031</td>
<td>0.102</td>
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<tr>
<td>IL-16</td>
<td>1.2</td>
<td>0.714</td>
<td>0.74</td>
<td>0.694</td>
<td>0.374</td>
<td>0.591</td>
<td>0.504</td>
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<tr>
<td>MIF</td>
<td>9.81</td>
<td>2.18</td>
<td>1.87</td>
<td>1.39</td>
<td>0.89</td>
<td>0.9</td>
<td>1.2</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>610</td>
<td>254</td>
<td>396</td>
<td>371</td>
<td>125</td>
<td>191</td>
<td>NDª</td>
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<tr>
<td>NT-proBNP</td>
<td>1.62</td>
<td>1.69</td>
<td>1.15</td>
<td>0.147</td>
<td>0.341</td>
<td>0.288</td>
<td>0.718</td>
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<tr>
<td>PAI-1</td>
<td>148</td>
<td>53.7</td>
<td>80.5</td>
<td>77.5</td>
<td>23.9</td>
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<tr>
<td>PDGF-BB</td>
<td>15.5</td>
<td>4.79</td>
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<td>75.9</td>
<td>61.1</td>
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<td>47</td>
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<tr>
<td>TGF-alpha</td>
<td>0.086</td>
<td>0.023</td>
<td>0.058</td>
<td>0.027</td>
<td>Lowª (&lt;0.006)</td>
<td>Lowª (&lt;0.006)</td>
<td>0.014</td>
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<tr>
<td>Thrombospondin-1</td>
<td>7280</td>
<td>5180</td>
<td>6700</td>
<td>4870</td>
<td>927</td>
<td>2120</td>
<td>4900</td>
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</table>

Fig. 2. suPAR levels during MSC treatment serum levels of suPAR (ng/mL) were analyzed in the following samples: pre-BM-MSC infusion I (T0), after BM-MSC infusion III (T-III), one year after the first infusion (T1y) and two years after the first infusion (T2y).

Please cite this article in press as: Belingheri M, et al., Allogeneic mesenchymal stem cell infusion for the stabilization of focal segmental glomerulosclerosis, Biologicals (2013), http://dx.doi.org/10.1016/j.biologicals.2013.09.004
draw any kind of definitive conclusions but in our opinion the persistent reduction of several inflammatory molecules is very biologically relevant and it would be of clinical interest to monitor this panel of factors in chronic kidney diseases as putative efficacious biomarkers during therapy.

After this encouraging single case, the hypothesis of a therapeutical effect of MSC in FSGS deserves to be tested in a prospective clinical trial in a large cohort of patients and further biological studies are needed to improve the full understanding of MSC mechanism of action in FSGS.

Conflicts of interest

All the authors declared no conflicts of interest.

Grant supports

The research has been partially founded by the EC FP7 through the Reborne project (Grant Agreement Number 241879) under the title “Regenerating Bone defects using new biomedical Engineering approaches”, and by grants from Associazione Italiana Ricerca sul Cancro (AIRC, Lombardia Molecular Imaging) and Ministero della Salute (Bando Cellule Staminali, Bando Giovani Ricercatori).

Contributions

MB and EG: patient care, clinical monitoring and samples collection; Ll: interpretation of data and manuscript editing; VP: cell biology assays, samples management and interpretation of data; EB, GG and AB: GMP cell production; RG, MPN and AE: manuscript revision; DC: suPAR analysis; PR: cell infusion and manuscript revision; LG: patient care, clinical monitoring, manuscript revision.

Acknowledgments

The authors thank Enrico Ragni and Elena Longhi for their molecular contribution, and Cristiana Lavazza for her iconographic support; and Fondazione Matilde Tettamanzi, Comitato Maria Letizia Verga, Comitato Stefano Verri, Fondazione MBBM and Fondazione La Nuova Speranza for their generous and continuous support. In addition, the authors thank the patient for his readiness to help others by allowing his case to be the focus of this article.

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