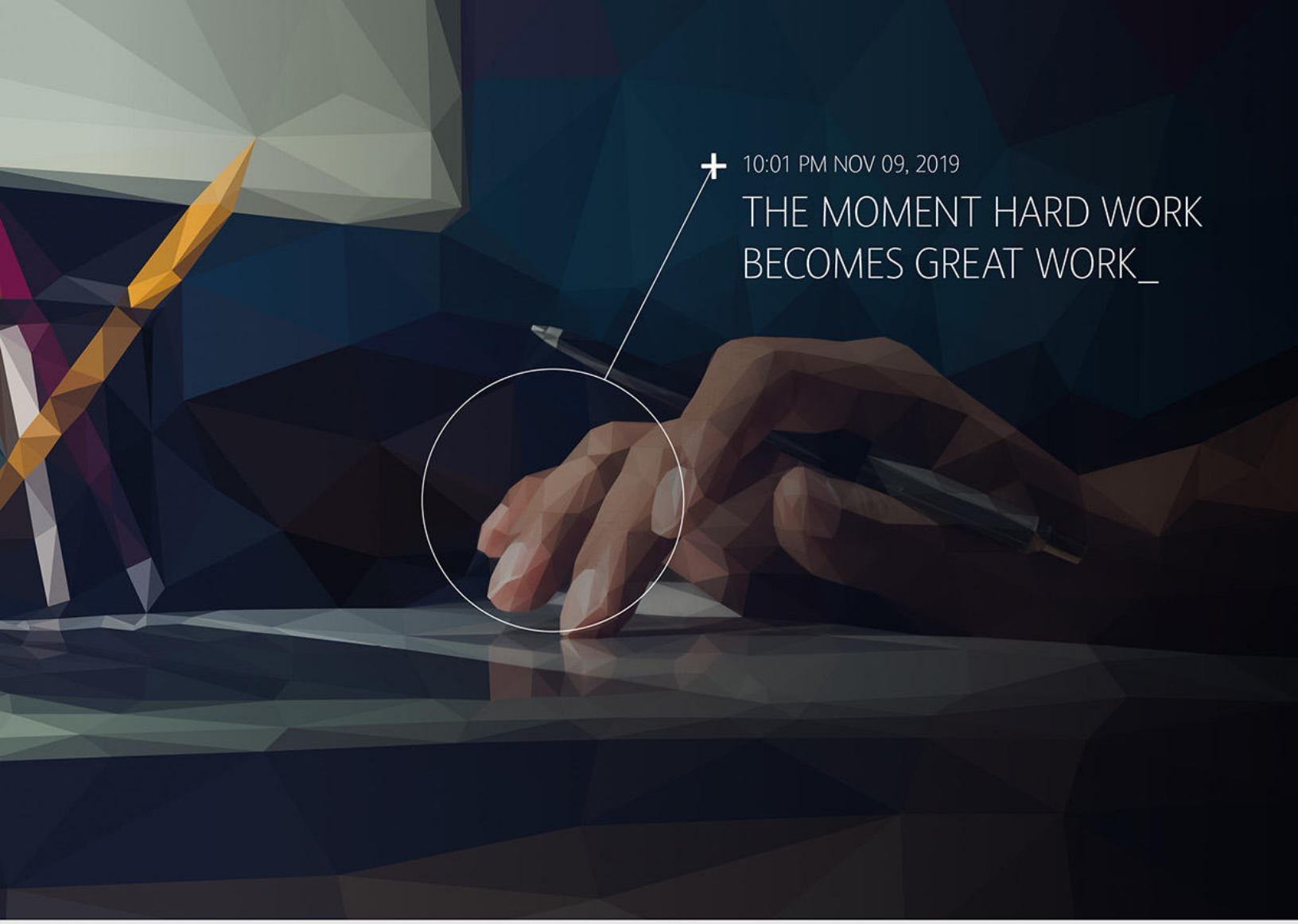

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Fetal Membrane Cells for Treatment of Steroid-Refractory Acute Graft-Versus-Host Disease

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Key Words. Fetal membrane cells • Decidua • Mesenchymal stromal cells • Graft-versus-host disease • Hematopoietic stem cell transplantation • Regenerative medicine

ABSTRACT

The placenta protects the fetus from the mother's immune system. We have previously found that fetal membrane cells (FMCs) isolated from term placenta prevent alloreactivity in vitro. FMCs share many features with bone marrow-derived mesenchymal stromal cells (MSCs), which we previously introduced to treat severe acute graft-versus-host disease (GVHD). Here, we tested FMCs for treatment of steroid-refractory acute GVHD. After two passages in culture, approximately 10^9 FMCs were obtained from one single placenta, although not all cells from passage 0 and passage 1 were used for expansion. The FMCs were positive for CD29, CD44, CD73, CD90, CD105, and CD49d but were negative for hematopoietic, endothelial, and epithelial markers. Microsatellite polymorphism analysis showed that FMCs were of maternal origin. All FMCs used showed normal karyotype. Nine patients who had undergone hematopoietic stem cell transplantation (HSCT) and who had developed steroid-refractory grade III–IV acute GVHD were given 0.9–2.8 × 10^6 FMCs per kg at 15 infusions. Median age was 57 years. There was no toxicity from infusion of FMCs in eight patients. One patient had seizures after infusion. Two of eight evaluable patients had a complete response and four had a partial response, giving an overall response rate of 75%. Two patients showed no response at all. Three patients are alive from 6 to 21 months after HSCT. One patient is well and two have chronic GVHD. Thus, FMCs may be successfully used for immune modulation and tissue repair. STEM CELLS *2013;31:592–601*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The placenta and the fetal membranes function as immunological barriers between the mother and the developing fetus during pregnancy. Maternal and fetal immune cells come into direct contact in the decidua, which is a membrane of maternal origin that plays an important role in fetomaternal tolerance [1, 2]. Stromal cells isolated from various parts of placental tissues, including amnion, chorion, decidua, and umbilical cord have recently been examined for their multipotent differentiation capacities and for their immunosuppressive capacities [3–7]. Intact fetal membranes from term placentas have been used for almost a century to treat severe burn injuries [8], and the amniotic membrane is also being used for repair of corneal ulcers [9]. Thus, the fetal membrane and the adjacent decidua could provide a valuable source of cells with regenerative and immunosuppressive properties that may be suitable for treatment of various inflammatory disorders.

Allogeneic hematopoietic stem cell transplantation (HSCT) is well-established for the treatment of leukemia, other severe disorders of the immune hematopoietic system, and some rare inborn errors of metabolism [10–12]. A major problem after HSCT is graft-versus-host disease (GVHD) [13, 14]. There is a need to improve therapy for severe steroid-refractory acute GVHD. We introduced bone marrow-derived mesenchymal

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stem cells (BM-MSCs) for treatment of therapy-resistant GVHD [15, 16]. Some patients have a dramatic response and others do not respond at all. The effect on GVHD has been confirmed using MSCs from various sources (adipose tissue, umbilical cord) that were expanded in fetal calf serum (FCS), platelet lysate medium, or human AB (blood group) plasma [17–19]. MSCs have low immunogenicity and immune modulatory effects, and they may be important for tissue repair [20–24]. MSCs express a variety of markers and can differentiate into cell types of mesenchymal origin, including chondrocytes, adipocytes, and osteocytes [25–27]. The sources of MSCs used in clinical trials have mainly been from BM or fat, but placenta may be a more readily accessible source of MSCs [28].

Stromal cells from term placental tissue and umbilical cord tissue share many features with BM-MSCs in terms of differentiation potential and surface marker expression [3, 5, 6, 29–31]. We have developed a protocol for generation of large quantities of fetal membrane cells (FMCs) from placenta with immunosuppressive and homing properties [4]. These cells are accessible without any invasive procedures and with little or no need for ethical considerations, as the placenta is normally discarded after delivery. The purpose of this pilot study was to investigate whether FMCs are useful in the treatment of steroid-refractory acute GVHD.

Patients and Methods

Preparation of FMCs

Human term placentas were obtained from healthy mothers during elective caesarean-section births, after obtaining informed consent. Ethical approval was obtained from the institutional ethical review board (2009/418-31/4). All donors were seronegative for HIV, hepatitis A and B, and syphilis. The placenta was put in a sterile container and immediately transferred to the laboratory for processing. The fetal membrane was carefully dissected from the placenta in a class-II biosafety flow-cabinet and transferred to a sterile beaker for extensive washing in Hyclone phosphate buffered saline (PBS) (Thermo Fisher Scientific, Waltham, MA, http://www.thermofisher.com). The tissue was cut into smaller pieces and transferred to 50-ml Falcon tubes (BD Biosciences, San José, CA, http://www.bdbiosciences.com). An equal volume of trypsin/EDTA (Thermo Fisher Scientific) was added to the tissue for 10 minutes at 37°C and was then discarded. The tissue was then incubated twice in trypsin/EDTA for 40 minutes at 37°C, and the trypsin digests were pooled and washed in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific) containing 10% FCS (Thermo Fisher Scientific) and penicillin-streptomycin (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) (hereon referred to as complete DMEM). The digested cells were counted and seeded at 2.9×10^5 cells per cm^2 in Nunc T175 flasks (Nunc A/S, Roskilde, Denmark) using complete DMEM. The remains of the fetal membrane were washed in complete DMEM and cut into 3–4-cm^2 pieces, and these were spread out and incubated in T175 flasks. The tissue explants were removed from the flasks when colonies of fibroblast-like cells appeared after approximately 1 week of culture.

When the cells from the trypsin-digested suspension and from the tissue explants were about 90%–95% confluent, the cells were harvested with trypsin/EDTA, washed in complete DMEM, and seeded in new T175 flasks at 2.9×10^3 cells per cm^2 in complete DMEM. The cells were cultured to passage 2 or 3 and frozen slowly in complete DMEM containing 10% dimethyl sulfoxide (DMSO) (WAK-Chemie Medical GmbH, Steinbach, Germany). FMCs were expanded and cultured under good manufacturing practice conditions using a room with reversed isolation, a sterile bench, and a separate incubator for cells from each donor.

Characterization of Cells

Data from FMCs were collected using a Gallios fluorescence-activated cell sorter (Beckman Coulter, Brea, CA). The data were analyzed using FlowJo software (TreeStar Inc., Ashland, OR).

For osteogenic differentiation, FMCs and MSCs were grown in complete DMEM supplemented with dexamethasone (0.1 μM), ascorbic acid (0.05 mM), and glycerophosphate (10 mM). The cells were stained for calcified structures using Alizarin red stain. For adipogenic differentiation, the cells were grown in complete DMEM supplemented with 1-methyl-3-isobutylxanthine (0.5 mM), dexamethasone (1 μM), insulin 10 (10 μg/ml), and indomethacin (0.2 μM). Adipogenesis was measured from the accumulation of neutral lipids in fat vacuoles, stained with oil red O.

The methods for mixed lymphocyte reactions (MLRs) have been described elsewhere [4]. Briefly, FMCs were added to MLRs at a ratio of 1:10, and proliferation was measured after 5 days by ^{3}H-thymidine incorporation. The data were analyzed by Wilcoxon signed ranked test (Graphad Prism software, San Diego, CA).

Analysis of FMC Origin

The origin of the FMCs was determined by examining microsatellite polymorphism [32]. DNA was extracted from FMCs, cord blood, amniotic epithelial cells, and blood from the donating mother using the EZ1 DNA blood kit and an EZ1 Advanced XL instrument (Qiagen, Hilden, Germany, http://www1.qiagen.com). DNA samples were amplified with different microsatellites and analyzed using capillary electrophoresis (ABI 3130XL Genetic Analyzer; Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com).

Karyotyping

Karyotype analysis with conventional CTG-banding of FMC cultures was performed using standard cytogenetic procedures. At least 11 metaphase nuclei from each sample were analyzed.

Patients

Between 2011 and May 2012, nine patients were treated with FMCs for acute GVHD (Table 1). Follow-up was on October 1, 2012. The study was approved by the Ethics Committee of Karolinska University Hospital, Huddinge. Patients and donors of FMCs gave written informed consent.

Conditioning

Three patients (numbers 1, 2, and 9) were conditioned with fludarabine (30 mg/m^2) for 5 days and busulfan 8 mg/kg (Table 1). The patient with aplastic anemia (number 3) was conditioned with fludarabine for 5 days and total body irradiation (TBI; 2 Gy/day) for 3 days. Patients 5 and 8 received fludarabine as above and treosulfan (14 g/m^2) for 3 days. A patient with Fanconi anemia (number 4) was given fludarabine and half-dose treosulfan. Patient 7 was conditioned with busulfan (16 mg/kg) and cyclophosphamide (120 mg/kg) [33]. The patient with lymphoma (number six) was conditioned with fludarabine for 6 days, cyclophosphamide (60 mg/kg) for 2 days, and TBI at 3 Gy/day for 2 days.

Prophylaxis of GVHD

Before HSCT, five recipients of matched unrelated donor grafts were treated with thymoglobulin (6–8 mg/kg; Genzyme, MN) [34]. To prevent GVHD, methotrexate (Mtx) was combined with cyclosporine (CsA) in six patients (numbers 2, 3, 4, 5, 7, and 9) [35, 36]. Patient 3 was given a modified protocol and Cy (50 mg/kg) on days +3 and +4 [37]. Three additional patients (numbers 1, 6, and 8) and the patient who received a cord blood transplant (number 8) were treated with tacrolimus combined with sirolimus [38]. The supportive care has been described in detail elsewhere [36]. Steroid refractoriness was defined as 8 days of high-dose prednisolone (2 mg/kg per day) without any response.

Grading and Treatment of Acute GVHD

Acute GVHD was graded from 0 to IV according to the grading by Glucksberg and coworkers from the Seattle team [39]. Acute

Table 1. Characteristics of patients treated with FMCs for acute GVHD

No.	UPN	Sex/age	Diagnosis	Donor	Acute GVHD, grade	Organ	FMC × 10^6/kg per passage	FMC, days after HSCT	FMC effect on GVHD	Outcome
1a	1462	F/64	MDS	HLA-id sib	III	GI-tract	0.9/2	113	NR	Home with TPN
1b					III	GI-tract	2.2/2	219	PR	Chronic GI-GVHD. TPN. Alive at 1 year 9 months.
2	1468	M/57	AML CR1	HLA-id sib	III	GI-tract	2.2/2	103	CR	Chronic GVHD skin. Alive and well at 1 year 7 months
3a	1504	F/53	SAA	MUD	III-IV	GI, skin	2.0/2	41	PR	Normal stool. Slight abdominal pain.
3b					III? + adeno	GI-tract	2.2/2	104	NR	Diarrhea. Adenovirus in blood and stool.
3c					IV? + toxicity	GI-tract, liver	2.8/2	131	NR	Bilirubin increased to 284. Diarrhea. Death from septicemia. Melena disappears.
4	1518	M/13	Fanconi, MDS	MUD	IV	GI-tract, liver	2.6/2	26	Not evaluable	GVHD, septic shock, death on day +37.
5	1517	M/58	CMML	HLA-id sib	III	GI-tract, skin	2.2/3	70	PR	Death. Relapse at 3 months.
6	1531	M/62	AML	MUD	III	GI-tract	1.7/3	31	PR	Septic shock, ICU, dialysis; cerebral hemorrhages; death on day + 83.
7a	1544	M/46	CML/MDS	MUD	III	GI-tract, liver	1.5/2	31	NR	Adenovirus in stool, increasing diarrhea
7b					IV	GI-tract, liver	2.0/2		NR	Increase in bilirubin, deterioration; death from GVHD on day +59.
8a	1555	M/10 mo.	LCH	CB unrel	III	GI-tract, skin	2.7/2	23	CR	Discharged on day +40.
8b					III	GI-tract	2.4/2	43	CR	Alive and well at 6 months.
9a	1547	F/62	Myeloproliferative disease	HLA-id sib	IV	GI-tract, melena, skin rash	2.2/3	108	NR	Melena stopped, but diarrhea and abdominal pain continued.
9b					IV	GI-tract	2.0/2	115	NR	Died from GVHD on day +171

Abbreviations: AML, acute myeloid leukemia; CB, cord blood; CML, chronic myeloid leukemia; CMML, chronic myelomonocytic leukemia; CR, complete response; CR1, first complete remission; FMC, fetal membrane cells; GI, gastrointestinal; GVHD, graft-versus-host disease; HLA-id sib, human leukocyte antigen identical sibling; HSCT, hematopoietic stem cell transplantation; ICU, intensive care unit; LCH, Langerhans' cell histiocytosis; MUD, matched unrelated donor; MDS, myelodysplastic syndrome; NR, nonresponsive; PR, partial response; SAA, severe aplastic anemia; TPN, total parenteral nutrition.

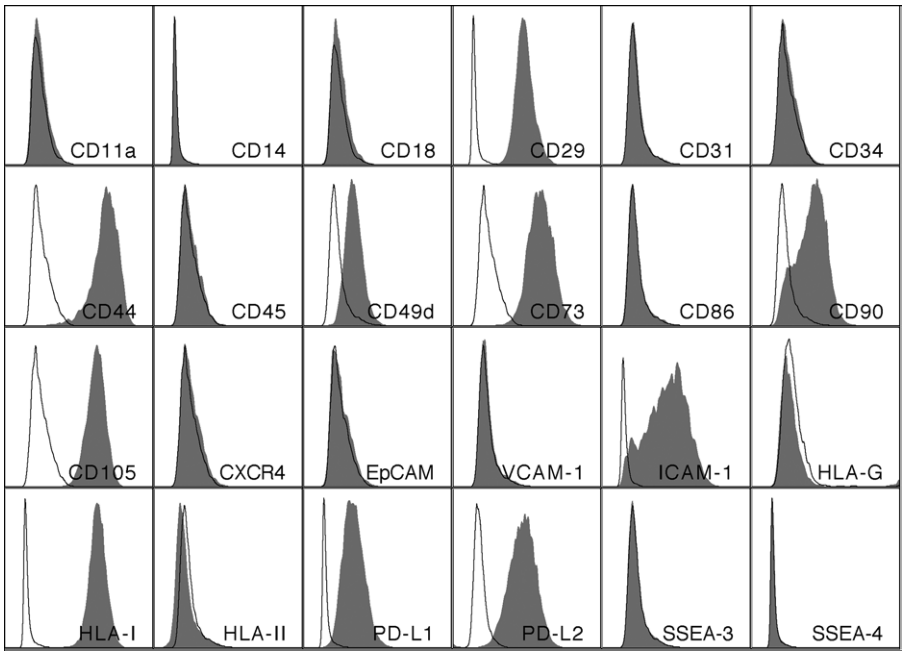

Figure 1. Phenotypic analysis of fetal membrane cells by flow cytometry. The histograms depict the expression of the different molecules (filled gray) compared to isotype controls (white) and shows one representative experiment out of four.

GVHD of grade I was treated with prednisolone per os, 1–2 mg/kg per day. Acute GVHD of grades II–IV was treated with prednisolone (2 mg/kg per day) and sometimes with Solu-Medrol (0.5 g i.v.). In addition, gastrointestinal GVHD was treated with oral budesonide (3 mg three times a day). Three patients (1, 5, and 9) were also treated with Mtx (5–10 mg/m^2) once a week for 1 or 2 weeks. Patients 7 and 9 received rapamycine. If the patients did not respond to steroids, they were given FMCs intravenously, aiming at a dose of 2×10^6 cells per kg.

Infusion of Cells

The cells were thawed at 37°C and diluted in CliniMACS PBS-EDTA buffer (AmCell Miltenyi Biotec GmbH, Gladbach, Germany, http://www.miltenyibiotec.com) supplemented with 5% AB-plasma (obtained from the local blood bank) in 50-ml Falcon tubes (BD). The cells were counted and resuspended in NaCl infusion solution (B. Braun Melsungen AG, Melsungen, Germany) with 5% AB-plasma at a concentration of 2×10^6 cells per ml. The solution was filtered through a 70-μm cell strainer (BD) before it was transferred to a heparinized syringe. All cell preparations were tested for bacterial contamination before and after thawing and also after preparation of the infusion suspension.

FMCs were infused intravenously for 5 minutes via a central venous line. Pulse, blood pressure, and well-being of the patient were checked regularly for 2 hours after infusion of the cells and noted in the charts.

Response

Response was evaluated independently by two clinicians (O.R. and J.M.). Complete response was defined as disappearance of all symptoms of GVHD. Partial response was defined as significant improvement ($\geq$1 grade), but signs of GVHD were still present.

Results

Expansion of Fetal Membrane Cells

The FMCs morphologically resembled BM-MSCs after 1–2 passages. Cells from four different placentas were isolated, expanded, and given to patients. From the first placenta, 9.5×10^8 cells at passage 2 and 1.0×10^9 cells at passage 3 were generated. For the three other placentas, a total of 1.2×10^9, $6.1. \times 10^8$, and 9.5×10^8 FMCs have so far been expanded to passage 2, and 3.4×10^8, 8.1×10^8, and 6.6×10^8 cells, respectively, were expanded to passage 3. When the culture flasks were 90%–95% confluent and harvested, $4–7 \times 10^6$ FMCs were obtained from each flask. Passage numbers of the cells and the amounts of cells per kg given to each patient are shown in Table 1.

Characterization of FMCs

The phenotype of the isolated FMCs was characterized by flow cytometry. The cells from both the trypsin-digestion method and the tissue explant isolation method were positive for the typical MSC markers CD29, CD44, CD73, CD90, and CD105 but negative for hematopoietic markers CD45, CD14, and CD34 and for the endothelial and epithelial markers CD31 and EpCAM-1, respectively (Fig. 1). Unlike BM MSCs, the FMCs were positive for both PD-L1 and PD-L2, and for the α4 integrin subunit (CD49d). Furthermore, FMCs were highly positive for ICAM-1 but were negative for CD86, human leukocyte antigen (HLA)-G, SSEA-3, and SSEA-4, and for CD11a, CD18, CXCR4, and VCAM. FMCs were positive for HLA class-I antigens but negative for HLA class-II antigens like BM-MSC [40] (Fig. 1).

Differentiation

FMCs showed some adipogenic differentiation. In general, they showed poor capacity to differentiate into osteogenic and adipogeneic lineages when grown in induction media, whereas control BM MSCs always differentiated into fat and bone under these conditions (data not shown).

Immunomodulatory Effects

The immunosuppressive effect of FMCs was examined in MLRs, which showed that the cells potently suppressed proliferation in an allogeneic setting (Fig. 2).

Origin of FMCs

The origin of FMCs was examined by microsatellite polymorphism, using cord blood and/or amniotic epithelial cells and maternal blood as controls. This showed that all cells used for transplantation were of maternal origin, indicating that the stromal cells were derived from the decidua parietalis (Fig. 3). This was further supported by karyotype analysis of the FMC lines derived from placentas taken at the birth of boys, which showed that the cells had two X chromosomes and were therefore of maternal origin.

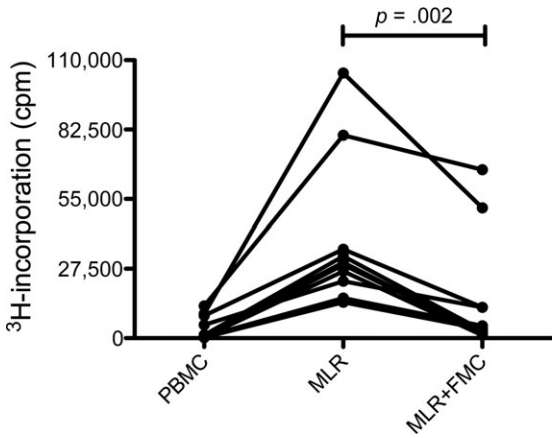

Figure 2. MLR from two separate experiments from each of the four placentas from which cells were isolated and infused into patients. When FMCs were added to the MLRs at a responder PBMC:FMC ratio of 10:1, the proliferation was significantly reduced compared to the control MLR (Wilcoxon signed ranked test). Abbreviations: FMC, fetal membrane cell; MLR, mixed lymphocyte reaction; PBMC, peripheral blood mononuclear cells.

Karyotyping

The karyotype of the FMCs was examined at passage 2 and at passage 10. Karyotype analysis showed normal female karyotype for all four FMC cultures.

Patient Outcomes

Patient 1 (UPN 1462). A 64-year-old woman developed diarrhea on day +97 after HSCT (Table 1). A colonoscopy showed grade 3–4 GVHD [41] of clinical grade-II to -III. After 10 days of treatment with steroids (2 mg/kg per day) with no response, she was treated with FMCs but with no obvious effect within a month. However, she was discharged after 5 weeks. Two months later, she was readmitted due to watery diarrhea and an acute GVHD of grade III. The steroid dose was increased, and she was again treated with FMCs. This time, over 2 weeks the watery diarrhea became less frequent: from five to seven times per day to twice a day. She was subsequently discharged to the outpatient clinic. She developed chronic GVHD with malabsorption and required total parenteral nutrition (TPN). She is off steroids but receives tacrolimus 1 year and 9 months after her transplant.

Patient 2 (UPN 1468). A 57-year-old man was readmitted 3 months after HSCT due to watery diarrhea up to 12 times a day. He was started on steroids (2 mg/kg per day). A colonoscopy showed GVHD of grade 2–3. Three weeks after readmission, he was treated with FMCs. Before cell therapy, his albumin was 16 g/l, and he required albumin infusions two to three times a week. After FMC infusion, diarrhea decreased and albumin increased to >20 g/l. Albumin is now normal: >38 g/l. More than one and a half years after transplantation, the patient is suffering from chronic GVHD of the skin. He

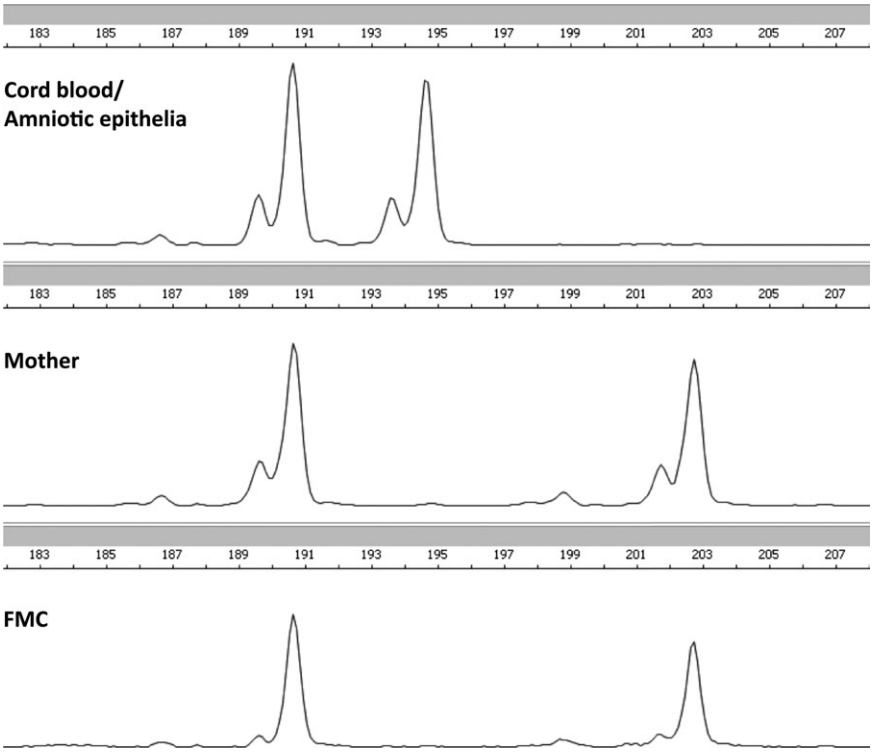

Figure 3. The stromal cells isolated from term fetal membrane were of maternal origin. This representative experiment shows the results of capillary electrophoresis of polymerase chain reaction products with primers targeting microsatellite DNA specific for the mother and child. DNA amplified from FMCs corresponded to that from the mother. Abbreviation: FMC, fetal membrane cell.

also has mild obstruction. He is being treated with CsA and prednisolone, 15 mg daily.

Patient 3 (UPN 1504). A 53-year-old woman with severe aplastic anemia developed acute GVHD of the skin and gastrointestinal tract on day 24. Gastroscopy showed grade 3–4 GVHD. After institution of prednisolone (2 mg/kg per day) and budesonid (3 mg three times daily) there was initial improvement, but on day +39 her GVHD worsened—with up to 2 l of diarrhea with melena. Two days later, she was given 2×10^6 FMCs per kg. Her albumin was 18 g/l. Improvement was seen within a week, and she could start to feed herself. She was discharged after 1 month, when she could feed herself and albumin had increased to 30 g/l. Prednisolone was decreased from 2 mg/kg per day to 0.5 mg/kg per day. She was given Mtx on two occasions. On day +99, she was readmitted due to severe diarrhea. An abdominal CT showed thickening of the small bowel, but the colon was normal. Colonoscopy did not reveal GVHD. On day +104 she was given FMCs. She was adenovirus-positive in stool and blood in polymerase chain reaction (PCR) (240,000 copies per ml). She was treated with cidofovir (300 mg at three occasions), and she needed TPN. Steroid dose was between 0.25 and 0.5 mg/kg per day. Due to marrow failure, she was given a booster bone marrow with 10×10^6 enriched CD34+ cells per kg and increasing white blood cell counts. Due to worsening of diarrhea and increase in bilirubin (92 mmol/l), FMCs were given on day +131. Abdominal pain worsened and bilirubin increased to 284 mmol/l. The patient developed septicemia and died 10 days later. Autopsy showed massive gastrointestinal hemorrhages and a pulmonary abscess.

Patient 4 (UPN 1518). A 13-year-old boy with Fanconi anemia and myelodysplastic syndrome (MDS) developed grade-IV acute GVHD of gut and liver with 279 mmol/l bilirubin. Due to deterioration, he was admitted to the intensive care unit (ICU) and infusion of FMCs was given. He had seizures during infusion, which were thought to be due to leukoencephalopathy. Two days after FMC infusion, all therapy was discontinued and palliative care was started. Five days after FMC infusion, hemorrhages from the gastrointestinal tract disappeared and due to the slight improvement, therapy with sirolimus was started. However, the patient deteriorated and expired of septic shock, probably due to GVHD on day +37. Bilirubin was 204 mmol/l.

Patient 5 (UPN 1517). A 58-year-old man with chronic myelomonocytic leukemia was readmitted on day +57 due to severe abdominal pain and melena. A colonoscopy showed grade-3 to -4 GVHD. He was treated with prednisolone (2 mg/kg per day) and a dose of Mtz. Due to nonresponsiveness, he was given FMCs on day +70. An abdominal computerized tomography showed swollen and thickened intestines. He had constant abdominal pain. After receiving FMCs, he had less diarrhea and less pain, and steroids were tapered. He had blasts in BM and died from relapse 3 months after HSCT.

Patient 6 (UPN 1531). A 62-year-old man with follicular lymphoma had vomiting and diarrhea. A gastroscopy on day +13 showed GVHD of grade 1–2. He was started on prednisolone (2 mg/kg per day) and budosemid. Due to increase in diarrhea despite negative findings from a rectoscopy, he was treated with FMCs on day +31. After this, he had partial response with no hemorrhages and less diarrhea. Due to septic shock from *Klebsiella* pneumonia, he was treated in the ICU from day +40. After a stormy course with multiple organ failure and dialysis, he was back on the ward after 40 days at the ICU. He died on day +83. Autopsy showed major cerebral hemorrhages and broncho-pneumonia.

Patient 7 (UPN 1544). A 46-year-old man with chronic myeloid leukemia and MDS had watery diarrhea on day +13. He started on steroids and budesonide. A colonoscopy on day +20 showed grade-2 GVHD. On day +25, he had adenovirus in stool and bilirubin levels started to increase. On day +31, he was given 1.5×10^6 FMCs. Bilirubin was 127 mmol/l. Adenovirus PCR was positive in blood (80–800 copies). His diarrhea continued and bilirubin increased to 396 mmol/l, at which time he received a second dose of FMCs, 2×10^6 per kg on day +39. He was treated with liposomal amphotericin B due to pulmonary infiltrates (probably fungi). He was also treated with extracorporeal psoralene and ultraviolet light and rapamune. Due to pulmonary emboli, he was treated with fragmin from day +28. He deteriorated and died on day +59 from severe acute GVHD with diarrhea; bilirubin was 456 mmol/l.

Patient 8 (UPN 1555). A 10-month-old boy with Langerhans' cell histiocytosis underwent a cord blood transplantation with a cell dose of 14×10^7 nucleated cells per kg. On day +20, he had vomiting and watery diarrhea (10 times in 24 hours). The diarrhea worsened, and he developed a skin rash on the back. High-dose prednisolone was given intravenously. On day +23, he received 2.6×10^6 FMCs per kg. He had normal stools from day 31. The skin rash disappeared. Because of recurrent diarrhea, he was treated with increasing doses of steroids and a second dose of FMCs on day +43. Nine days later, the feces was normal and he was discharged 2 weeks after the second dose of FMCs.

Patient 9 (UPN 1547). A 62-year-old woman with myeloproliferative disorder had vomiting on day +19. Prednisolone was started, and a gastroscopy showed acute GVHD of grade 1–2. She improved and was discharged. She was readmitted because of diarrhea, melena, and severe abdominal pain on day +103. She did not respond to steroids and could not eat due to immediate severe pain and diarrhea even after minimal oral intake. Gastroscopy showed GVHD of grade 2–3. She also had a skin rash on the head and on the breast. She was given FMCs on day +108. She was unable to take the prescribed oral budesonid. Melena disappeared, but the abdominal pain and diarrhea continued. She was therefore given a second dose of FMCs on day +115. She required frequent albumin infusions. Mtx and later rapamune were added. She was sent home for palliative care and died on day +171.

Overall Response and Survival

Of the eight patients with acute GVHD who could be evaluated, two (numbers 2 and 8) had complete responses and four patients (numbers 1, 3, 5, and 6) had partial responses, with a total response rate of 6/8 (75%). Patient 3 (UPN 1504) had a partial response to the first FMC dose. She did not respond to the two following doses, when she had adenovirus infection and cidofovir toxicity, respectively. However, recurrence of acute GVHD in the small bowel cannot be ruled out. Patient 9 did not respond to two doses, although hemorrhaging disappeared after the first dose. The complete nonresponse rate was 2/8 (25%). Patient 4 was given FMCs in the ICU and due to poor conditioning and deterioration, palliative care was instituted. Immunosuppressive drugs were discontinued. Although hemorrhaging stopped, he was considered unevaluable for this GVHD therapy. Melena stopped in three patients (numbers 3, 4, and 9) after treatment with FMCs. Patient 5

died due to relapse. When the relapse was confirmed, chimerism analysis of blood samples performed before infusion of FMCs was done, indicating that he had relapsed before infusion of FMCs.

Three of the nine patients are alive from 6 months and up to 1 year and 9 months after HSCT. Patients 2 and 8 had complete responses and are well, but patient 2 has chronic GVHD. Patient 1 suffers from malabsorption and chronic GVHD.

Discussion

MSCs have been used for the repair and regeneration of a variety of organs and tissues. Most often, MSCs have been used for treatment of acute GVHD [12, 15–19, 27]. In addition, MSCs have also been explored for treatment of inflammatory bowel disorders, multiple sclerosis, autoimmune thrombocytopenic purpura [42, 43], and Crohn's disease [44–48]. They have also been used for wound healing [49, 50]. In HSCT, MSCs have been successfully used to enhance engraftment and for graft failure [51–55]. It may be logical to use BM-derived MSCs to enhance engraftment, because these cells reside in the BM and support the growth of hematopoietic cells and also produce factors of importance for hematopoiesis [27]. In the treatment of injuries caused by allogeneic cells, such as rejection and GVHD, it would seem rational to try FMCs, because these cells are important for protection of the fetus from the mother's immune system [1, 2]. Furthermore, we found that FMCs had a significantly stronger inhibition of MLR compared to BM-MSCs [4].

To our knowledge, FMCs have not been used previously to treat acute GVHD. The cells were safe to infuse and, as with MSCs, no acute toxic effects were seen in eight of the patients. The only patient who showed any form of reaction after infusion of FMCs was the young boy in the ICU (patient 4, Table 1). He had seizures after infusion of the cells. However, this was probably not due to infusion of the cells but rather due to leukoencephalopathy or CsA-induced neurotoxicity. Human placenta-derived MSCs have been infused prior to transplantation in one HSCT patient previously [56].

When we set up the protocol for generation of FMCs, we use intact fetal membranes, including the fetal amnion and chorion and the maternal decidua parietalis, but all lines generated for this study were solely of maternal origin. This indicates that the maternal decidual stromal cells have superior expansion capacities compared to fetal cells from the amnion and chorion. This is in line with a previous study showing that decidual stromal cells have better growth properties compared to amnion-derived stromal cells [57]. Our experience is also that both epithelial and mesenchymal cells from amnion show inferior growth kinetics compared to decidual cells (unpublished observations), and we further found that the immunosuppressive effect of FMCs were better than that of amnion-derived stromal cells [4]. Thus, based on better expansion capacities and the strong immunomodulatory effects, we decided to use these cells for treatment of acute GVHD. Fetal-derived cells may have better differentiation abilities, which may make them better candidates for regenerative purposes. Amnion-derived stromal cells have previously been described not only to differentiate into mesenchymal lineages but also into hepatocytic [58], myogenic [29], pancreatic [59], angiogenic [29], and neurogenic [30] lineages.

The overall response rate in our patients with acute GVHD was 75%, which is similar to that reported in patients treated with BM-derived MSCs for severe acute GVHD [15–19, 27]. To determine whether FMCs or BM-derived MSCs are most effective—or equally effective—for treatment of acute GVHD would require large, prospective randomized studies. If they are equally effective, FMCs have considerable advantages, as the placenta is discarded after delivery whereas aspiration of BM from volunteer donors is needed to obtain MSCs. FMCs are also easier to culture and they can therefore be used at an earlier passage than BM-MSCs, where several passages are needed to obtain sufficient numbers for therapy. A recent long-term analysis in our patients treated with BM-MSCs for acute GVHD showed that regardless of age, patients who received early passage MSCs (from passage 1 or 2) had significantly better survival than those who received MSCs from passage 3 or 4 [60].

The two patients with complete response (numbers 2 and 8) are doing well, which is in line with a larger series of patients treated with BM-MSCs, where those with complete response had 50% survival and those with partial or no response has survival of around 10% [16]. Such dramatic responses are never seen with immunosuppressive drugs, but we also saw this when we started to use BM-MSCs for severe acute GVHD [15, 16]. However, there were few patients in the trial and there was no control group treated with placebo. Therefore, it is not completely confirmed that the positive effects seen is due to FMCs, although there is no other explanation. Even if some of the responders were treated with increment of steroid dose and Mtx, such treatment cannot explain the positive findings seen in some of the patients. Of course, caution has to be given because of the small number of patients included in this pilot trial. The only way to determine the role of FMCs for severe acute GVHD is to perform a prospective multicenter randomized trial. A multicenter approach is necessary because each center have too few cases with steroid-refractory acute GVHD to accrue sufficient number of patients.

In this study, only two children were included. One of them (patient 4) had therapy-resistant acute GVHD, but due to poor performance all immunosuppressive therapy was discontinued. He could not be evaluated, because stromal cells probably also need immunosuppressive therapy to be effective for GVHD [61]. Of 151 MSC-treated patients described in the literature, the overall response rate was reported as 73% with a complete response rate of 52% [62]. This is encouraging because of the dismal outcome of steroid-refractory acute GVHD. However, the true response rate is probably slightly worse, because there is bias in the literature and those with poor outcome tend not to be reported to the same extent as those with good outcome.

We previously reported that children had a better response rate (84%) than adults (60%) [16]. The low complete response rate in this study, only 2/8, may have been due to the fact that most patients with steroid-refractory acute GVHD in this trial were adults of high age. Median age of the patients in this study was 57 years, as opposed to 22 years of age in our previous report, in which 45% were children with a complete response rate to MSCs of 68%. Using MSCs expanded in platelet lysate medium, 2 of 13 adult patients (15%) with steroid-refractory acute GVHD had a complete response [17].

BM-MSCs were successfully used to treat hemorrhaging after HSCT, such as hemorrhagic cystitis and major gastrointestinal bleeding [63]. BM-MSCs have also been reported to affect coagulation, especially at higher passages [64]. Interestingly, melena stopped in three patients with severe gastrointestinal GVHD after infusion of FMCs (patients 4, 6, and 9; Table 1). It remains to be determined whether FMCs, like MSCs, affect the coagulation system.

Like BM-MSCs, FMCs probably home to damaged tissue [65–67]. We also could demonstrate MSC donor DNA in abdominal lymph node and colon in a patient with acute GVHD

and in the bladder during hemorrhagic cystitis [15, 68]. Even if we have not proven that FMCs home to damaged tissue, it is likely that these cells mimic BM-MSCs in this respect. Although FMCs and BM-MSCs have several features in common, there are some differences that may be of importance. FMCs express a higher level of adhesion markers including CD29 (β1), CD49d (α4), and CD54 (ICAM-1) [4]. This may be of importance for homing when the cells are infused into the body.

The effect of using FMCs or MSCs on severe gastrointestinal GVHD is probably due to dual mechanisms. First, there is an immunosuppressive effect inhibiting alloreactivity. Regarding the immunosuppressive effect, FMCs and BM-MSCs appear to differ in the respect that the former require direct contact to inhibit MLRs, whereas BM-MSCs suppress alloreactivity even in a transwell system (Erkers et al., unpublished data) [69]. It is possible that both contact-dependent and contact-independent interactions are required, probably in a sequential manner [70]. BM-MSCs probably exert their immunosuppressive effects via several soluble factors. Factors that may be of importance include indoleamine 2,3-dioxygenase, interleukin (IL)-10, interferon-γ, prostaglandin E2, transforming growth factor β1, hepatocyte growth factor, IL-2, galectin-1, and nitric oxide [27]. The second effect may be interaction of MSCs with macrophages that may infiltrate the damaged gut. Stromal cells may attract proinflammatory macrophages with antimicrobial effects [71]. In line with this, paracrine factors of MSCs recruit macrophages and endothelial lineage cells, thus enhancing wound healing [49]. Through prostaglandin E2, MSCs polarize macrophages into anti-inflammatory macrophages, which promote wound healing [72, 73].

Six of the patients died, but three are still alive. The reason for the relatively high death rate is due to the fact that when a new therapy is tried, the first attempts are made in patients with a dismal prognosis. Patients with severe acute GVHD have a very poor outlook, with a high incidence of death due to infection and hemorrhaging [74]. When MSCs were given for acute GVHD, the response rate was much higher when they were given at an earlier stage for grade-II acute GVHD [19, 75]. In the latter study, after treatment of acute GVHD of grade II with MSCs, there was no death from acute GVHD in pediatric HSCT recipients. Therefore, early treatment using FMCs and MSCs for moderate acute GVHD may be a better way of preventing death from GVHD than waiting until the patients are steroid-refractory, as was done in this study using FMCs and in our previous studies using MSCs [15, 16, 60].

The Osiris Company have patent on BM-MSCs for a large number of clinical indications. This makes it impossible to use such cells expanded in research laboratories for clinical studies in the U.S. FMCs is a different type of cells with different features and they open the possibility for clinical stromal cell research at U.S. institutions.

Conclusions

To conclude, FMCs are readily available from deliveries after caesarian section. They are easy to expand, they express a high level of adhesion markers and inhibitory ligands, and they are highly suppressive in vitro, and thus may have an advantage over BM-MSCs. FMCs seems safe to infuse intravenously to most patients. The overall response rate in severe refractory acute GVHD appears to be similar to that seen when using BM-MSCs. Of course, caution has to be taken because of the small number of patients treated with FMCs, so far. This pilot study should encourage further clinical trials using FMCs. Indications for pilot studies might be where immune modulation and tissue repair are required, such as acute and chronic GVHD, organ allograft rejection, and autoimmune disorders, for example, ulcerative colitis or multiple sclerosis. This study may also provide a basis for further controlled studies using FMCs for acute GVHD.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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