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Research Article

Induction of Immune Tolerance towards Allogeneic Cells using Fetal Directed Placental Injection in a Murine Model

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ABSTRACT

Objective: In utero exposure to foreign antigens prior to the development of the immune system induces immune tolerance. We aimed to induce immune tolerance towards allogeneic cells by early gestational transplacental injection under ultrasound guidance in a murine model.

Methods: Bone marrow cells from C57BL/6-Green Fluorescence Protein transgenic mice were transplanted into the placenta of Balb/c fetal mice at 11-day of gestation under ultrasound guidance. Each fetus was injected with 2×10^5 cells/2.5 μl. After birth, we evaluated the immune response against allogeneic donor cells.

Results: The birth survival rate was 21.2% for allogeneic mice. Survival of the donor skin graft was 75% and successful in mice injected with fetal transplacental cells, whereas the transplanted allogeneic skin was all rejected within 4 weeks in control naïve mice (p=0.007). Cytotoxic immune reactivity against the allogeneic cells was suppressed according to the ELISPOT assay (p=0.002).

Conclusion: We showed that early transplacental allogeneic cell injection can induce donor-specific tolerance sufficient to allow tissue graft.

Keywords: Placental transplantation; Allogeneic model; Immune tolerance; Fetal stem cell transplantation; Chorionic villus sampling; ELISPOT assay; Skin graft

INTRODUCTION

Fetal therapy is important in preventing lethal disease and also for diseases that can cause permanent organ damage before birth. If postnatal treatments are limited to palliative options, fetal therapy including intrauterine stem cell therapy may be offered [1-3]. When allogeneic transplantation is performed in adults, the vigorous immune response against the transplant becomes the major problem. Intensive immunosuppression and myeloablation is required to prevent rejection or graft versus host disease. Therefore, immunologic tolerance is an important issue for stem cell transplantation success.

Immunologic tolerance is defined as unresponsiveness to an antigen that is induced by previous exposure to that antigen. In utero, the immune system is immature [4-7]. The fetus has been shown to accept allogeneic antigens, which introduces immune tolerance. During this period, stem cell transplantation is expected to result in successful engraftment without the need for myeloablation or immunosuppression. This reduces the risk of rejection reaction and myelosuppression. There have been previous studies, which have used in utero-transplantation in animal models [1,8,9]. Many of the animal disease models were successful in achieving a clinically significant level of chimerism [10]. In humans, stem cell transplantation in the fetus has been

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attempted for a variety of diseases [1,11,12]. However, in utero bone marrow cell transplantation has only been successful in immune deficiency states, where donor cells have a competitive advantage [11]. Recently, mesenchymal stem cell transplantation for osteogenesis imperfecta has resulted in a degree of clinical efficacy. However, these cases required both prenatal and postnatal transplantation [13,14]. Despite successful results from fetal transplantation in animal models, results for human fetus transplantation have been limited.

The development of the immune system is different in mice and humans, so the timing of when to induce immune tolerance is also different. In humans, the window for tolerance induction is thought to be limited to the first trimester, ending approximately 14 weeks after gestation [2-4].

Chorionic Villus Sampling (CVS) is widely utilized for prenatal diagnosis. It is performed at between 10 to 14 weeks of gestation. The technique used for CVS is an attractive approach for delivering cells and/or foreign antigens to the fetus since it may be possible to achieve fetal tolerance at this appropriate time. We previously reported an early gestational placental injection of donor cells carrying a foreign protein into a fetal murine model, which induced immune tolerance against the foreign protein [15].

In this study, we utilized the same procedure to induce immune tolerance with early gestational stem cell transplantation into the placenta in allogeneic murine models.

MATERIALS AND METHODS

Ethical statement

All procedures in this study were carried out in strict accordance with the guidelines for animal experimentation from the Animal Research Committee of Osaka University and that of the National Cerebral and Cardiovascular Center. The protocol was approved by the Animal Research Committee, Osaka University (Permit Number: 24-079-018), and National Cerebral and Cardiovascular Center (Permit Number: 13018). All surgery was performed under anesthesia, and all efforts were made to minimize suffering where possible.

Mouse recipients and donors

Eleven-day old embryos from Balb/C mice were designated as recipients in the allogeneic models. We used surrogate mothers by removing the influence of postnatal exposure to maternal alloantibody in breast milk [16]. Donor cells were harvested from C57BL/6TgN (act-EGFP) OsbY01 mice (kindly provided by Dr. Okabe, Osaka University, Genome Information Research Center) that had been maintained in our breeding colonies. Injected mice were housed in the Laboratory Animal Facility at the National Cerebral and Cardiovascular Center Research Institute. The experimental protocols were performed with approval from the Institutional Animal Care and Use Committees of the National Cardiovascular Center Research Institute.

Preparation of donor BMCs

Adult GFP-positive BMCs (B6GFP-BMCs) were isolated from B6GFP mice by flushing the ilium, humerus, tibia, and femurs with Ca/Mg-free Phosphate-Buffered Saline (PBS) using a 26-gauge needle. After filtration through a 40 μm mesh filter, B6GFP-BMCs were centrifuged at 440 xg for 5 minutes at room temperature. After the red blood cells were lysed with lysing buffer, the B6GFP-BMCs were counted and Suspended in PBS at a density of 8×10^7 cells/ml for injection.

In utero intra chorionic villi injection (ICVI)

In utero injections were performed at 11 days of gestational age using an ultrasound-guided injection system (Vevo 2100, VisualSonics, Toronto, Canada). We used the techniques developed and published by colleagues [15] to inject B6GFP-BMCs. Recipient mice were anesthetized with isoflurane (3.5% for induction, 2% for maintenance) and the abdomen was opened by midline laparotomy. One horn of the uterus was exteriorized and covered in pre-warmed sterile ultrasound gel (Aquasonic, Parker Laboratories, Fairfield, New Jersey, USA). The fetal mice were scanned using an 80MHz probe. The murine placenta consists of two layers; the estimated labyrinth layer and the estimated maternal decidua. The labyrinth layer represents the placental villi in humans and is the fetal side of the placenta [17,18]. It is possible to clearly identify each layer using ultrasonography. Pulled and beveled glass micropipettes (diameter, 75 μm) were loaded with B6GFP-BMCs. The micropipette tip was physically inserted into the placenta and a volume of 2.5 μl of 2×10^5 viable B6GFP-BMCs was injected into the estimated labyrinth layer of the placenta (Figure 1). We defined cell transplantation into the estimated labyrinth layer as ICVI. After injections, the uterus was placed back into the abdominal cavity and the abdominal incision of the dam was closed with 3-0 vicryl continuous suture.

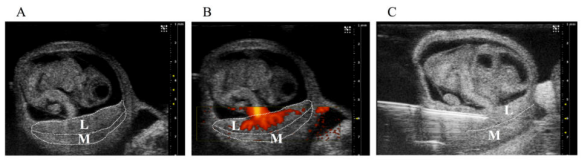

Figure 1: Ultrasound imaging and CVS technique in the mouse. A: We can identify the fetus, umbilical cord and two layers of placenta; B: The two layers were clear in B-mode with power doppler mode. The feto-placental blood flow was visible in the labyrinth layer, but not in the maternal decidua; C: The micropipette tip was physically inserted into the estimated labyrinth layer of the placenta (L: Labyrinth layer, M: Maternal decidua).

Skin grafting

Full-thickness tail skin from B6GFP mice was cut into 10 × 10 mm squares. The excised tail B6GFP skin was grafted on to the back of recipient mice 6 wk after birth, as previously described [15]. The graft was secured with a bandage for 7 days. Transplanted GFP-skin graft integrity was evaluated under UV light after skin grafting [19].

T-cell assay: enzyme-linked immunospot (ELISPOT)) assay

CTL activity was evaluated by ELISPOT assay. ICVI mice and naïve mice were repeatedly immunized by intraperitoneal injection with 100 μl of B6GFP-splenocytes as the immunogen at both 10 and 11 weeks post-birth. Two weeks after the last immunization, mice were anesthetized and splenocytes were harvested from isolated spleens by passage through a sterile strainer. The splenocytes were then sedimented by centrifugation at 440 xg for 5 minutes and red blood cells were depleted using ACK buffer for 10 minutes. The B6GFP-BMCs were harvested, sedimented by centrifugation at 440 xg for 5 minutes at room temperature, before re-suspension in RPMI 1640, and incubated with Mitomycin C for 20 minutes. Finally, isolated splenocytes (5×10^6/ml) were co-cultured with B6GFP-BMCs (5×10^5/ml) in 58 cm^2 BD Falcon dishes with recombinant human IL-2 at 37℃. After *in vitro* sensitization by the B6GFP-BMCs (the stimulator) for 4 days, the effector splenocytes and B6GFP-BMCs were seeded on to 96 well tissue culture plates (Millipore Ireland B.V, Germany), which was coated with anti-IFN-γ specific primary antibodies at 37℃ for 48 hours. The secreted IFN-γ was detected as spots by staining with phosphatase-labeled secondary antibodies and BCIP/NBT solution. The number of spots in each well was counted.

Statistical analysis

Details of statistical tests applied to each data set are given in the figure legends. Statistical comparison between groups was performed using a Wilcoxon test. The calculation for p-value was performed using JMP Pro11.2.1 (SAS Institute Japan). $p<0.05$ is considered significant.

RESULTS

The survival rate after transplacental injection

Eleven-day old embryos were obtained from 14 pregnant Balb/c dams, a total of 104 fetal mice, and were injected with B6GFP-BMCs. The assessment of the birth survival rate for this procedure was 21.2% (22 out of 104).

Skin graft survival and cellular immune tolerance induction against allogeneic cells

We evaluated whether the ICVI of allogeneic BMCs can induce immune tolerance against allogeneic cells. First, ICVI mice injected in utero with 2×10^5 allogeneic B6GFP-BMCs were transplanted into the tail skin from an allogeneic B6GFP transgenic mouse (C57BL/6 background). Successful engraftment of the transplanted allogeneic skin was observed in 75% (6 out of 8) of mice treated with ICVI 5 weeks after grafting, whereas the transplanted allogeneic skin in control Balb/c mice was completely rejected within 4 weeks (p=0.007) (Figure 2).

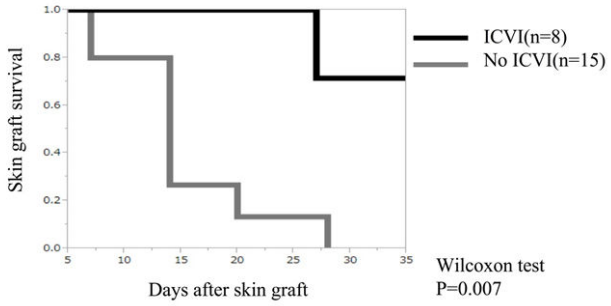

Figure 2: Skin graft survival: Kaplan-Meier analysis. The gray line shows the survival rate of allogeneic skin graft in ICVI mice (N=8) and the black line shows the survival rate in No ICVI mice (N=15). All skin grafts were rejected by mice, which had not received ICVI, in the 4 weeks after grafting. ICVI: Intra Chorionic Villi Injection.

Cytotoxic immune reactivity of host T cells against allogeneic cells was significantly reduced in mice undergoing ICVI as assessed by ELISPOT assay (p=0.002). The mean ± SE of the ICVI group was 26.7 ± 5.69, whereas the No ICVI group was 43.0 ± 4.30 (Figure 3).

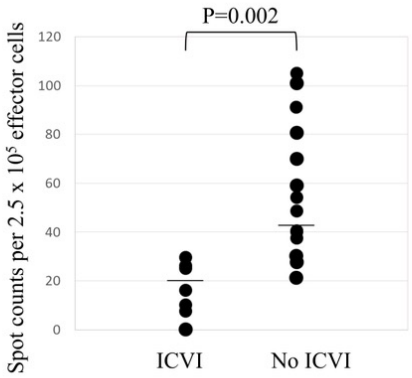

Figure 3: Cytotoxic T-cell assay against allogeneic cells: Wilcoxon test. CTL reactivity against allogeneic cells was measured using ELISPOT with indicated counts per 2.5×10^5 effector cells. ICVI mice (N=8) had lower responses than control mice, which had not received ICVI (N=15). ICVI: Intra Chorionic Villi Injection.

These data suggested that ICVI in fetal mice with allogeneic B6GFP-BMCs is sufficient to induce immune tolerance against allogeneic cells.

DISCUSSION

In this study, we have demonstrated that allogeneic cells were transplanted into fetal mice by intra-placental injection at early

gestational stage using an ultrasound-guided injection system and induced allogeneic immune tolerance.

There has been great progress in the technologies for prenatal imaging and molecular diagnostics available for prenatal diagnosis and prenatal therapy. The first report of exposure to foreign antigens can lead to tolerance was reported in 1945, using monochorionic dizygotic cattle that the shared placental circulation enabled exchange of circulating cells and resulted lifelong chimerism and donor-specific tolerance [20]. The first successful fetal transplantation in human was reported in 1989, using human fetal liver cells for transplantation into a fetus with bare lymphocyte syndrome [12]. The benefits of this treatment include perinatal rescue of an affected fetus with a life threatening disease, phenotypic cure, avoidance of irreversible organ damage and avoidance of postnatal treatment related complications in non-perinatally lethal disease. The smaller cell dose required for fetal therapy and generation of central tolerance to donor cells through establishment of prenatal microchimerism facilitates repeated postnatal transplantation [1]. As the immune system is immature during early fetal development, stem cell transplantation for the fetus can provide not only treatment but also induction of immune tolerance. In utero stem cell transplantation has been reported in several mice models, and these results suggested therapeutic potential for hemoglobinopathies, osseous diseases, skin diseases, inborn metabolic diseases and others [1]. However, in humans, the clinical efficacy of fetal stem cell transplantation was so far limited. To solve this problem, many studies have been conducted including donor cell selection, dose, mode, route, improving donor cell engraftment, and manipulating the microenvironment to facilitate homing [1]. We focused on the timing of transplantation to induce immunological tolerance in utero. In the early phase of gestation, the immune system undergoes a process of self-education. This occurs primarily in the fetal thymus and consists of two components. The positive selection of pre-lymphocytes for recognition of "self" Major Histocompatibility Complex antigen (MHC) and the negative selection of pre-lymphocytes that have high-affinity recognition of self-antigen in association with self MHC. The introduction of foreign cells prior to completion of this process of thymus education result in donor-specific immune tolerance [6,7]. Development of the immune system differs between humans and mice. Some form of immune tolerance can be induced with exposure to foreign antigen as late as 1 week after birth in mice. However, the human immune system begins to develop from 10 weeks gestation with emergence of mature T-cells after 13-14 weeks gestation and NK cells may pose a barrier as early as the end of the 1st trimester [2-4]. It may be necessary to utilize ICVI during the 1st trimester as this is feasible with CVS, in order to enhance the probability of induction of immune tolerance in humans.

Takahashi et al. demonstrated immune tolerance induction using intra-placental bone marrow cell transplantation in early gestational congeneic mice models [15]. Fleischman et al. demonstrated that hematopoietic engraftment and chimerism after intra-placental injection of hematopoietic cells [14]. Both of these were congeneic models [16,21].

The main limitation of this study is the differences in the placental structure and the development of the murine and human immune system. The fetal survival of allogeneic models was lower than in congeneic models. This was related to the difference between mouse strains and the influence of maternal immune reaction for allogeneic cells. Nijal et al. reported maternal T cell trafficking after in utero intervention and limited engraftment. Merianos et al. reported maternal allo-antibodies did not appear until approximately two weeks after in utero stem cell transplantation. After the postnatal period, maternal allo-antibodies transport implicating maternal breast milk. In this study, we used the surrogate mothers to prevent minimal influence of maternal immune reaction. The long-term effect of such treatments is unknown. The mechanism of immune tolerance does not examine in this study and therefore further study is necessary.

CONCLUSION

In conclusion, we utilized a technique similar to CVS to transfer donor cells carrying allogeneic cells into the fetal side of the placenta in the mouse models. This approach proved sufficient for induction of immune tolerance against the allogeneic cells in a murine model.

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CONFLICT OF INTEREST

The authors report no conflicts of interest concerning the materials or methods used in this review or the findings specified in this paper. The authors have no competing financial interests related to this study.

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