Induction of hemopoietic chimerism in the caprine fetus by intraperitoneal injection of fetal liver cells

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Summary. Intraperitoneal injection of allogeneic liver cells from 43-day-old male fetuses into normal 60-day female goat fetuses resulted in persistent hemopoietic chimerism in surviving recipients without clinical evidence of graft-versus-host disease. Transplantation of normal fetal liver cells into preimmunocompetent goat fetuses affected with β-D-mannosidosis may provide an alternative strategy for evaluating hemopoietic stem cell transplantation in the treatment of human lysosomal storage diseases.

Key words. Transplantation; goat; fetal liver; β-D-mannosidosis; hemopoietic chimerism.

The ability to induce hemopoietic chimerism in experimental animals without recourse to physical (i.e., irradiation) or pharmacological manipulation of the immune system, is important to our understanding of normal prenatal development of immunocompetence. It also provides a tool for exploration of the possible role of bone marrow transplantation (BMT) in the treatment of certain human diseases.

Our interest in the use of goats affected with β-D-mannosidosis [1, 2] as a model for studies on the treatment of lysosomal storage diseases (LSD) by BMT, stimulated us to investigate the induction of hemopoietic chimerism in this species. The studies reported here indicate that goats are similar to sheep in this respect.

Materials and methods. Normal goats (*Capra hircus*) from the research herd were used for breeding and transplantation experiments. Donors of bone marrow were normal adult goats. Bone marrow was obtained by multiple aspiration of the sternum and collected into culture medium (Medium 199 Formula No. 78-0496 Gibco Europe), containing preservative-free heparin 40 U/ml. After collection, the bone marrow was filtered and cryopreserved using dimethyl sulfoxide (DMSO, Sigma Chemical Co., St. Louis, MO, USA) as cryoprotectant. DMSO diluted to a final concentration of 20% with the same medium, was added to the bone marrow suspension over a 5-min period until a 1:1 ratio was obtained. The bone marrow/DMSO suspension was frozen in aliquots of 1.5 ml with the aid of a programmable biological freezer (Chamber model 10–16 and controller model 10–20, Planer Products Ltd., Sunbury-on-Thames, Middlesex, U.K.) at a rate of − 1 °C/min to − 30 °C, then − 2 °C/min to − 60 °C, and transferred to liquid nitrogen for storage at − 196 °C. For the transplantation experiments, cryopreserved bone marrow was thawed rapidly at 37 °C and Ringer's lactate at 4 °C was added to a final ratio of 1:1. The resultant cell suspension was centrifuged at 1000 rpm for 10 min and the pellet resuspended in 0.5–0.8 ml of Ringer's lactate. Viability of the thawed final preparation was 36% (female donor) and 42% (male donor) assessed by trypan blue exclusion.

Fetal liver hemopoietic cell suspensions were prepared essentially according to the technique of Flake et al. [3]. Livers were harvested from 43-day-old fetuses, trimmed of connective tissue and transferred to heparinized (preservative-free 10 U/ml) α-minimum essential medium (MEM) containing 10% goat serum at room temperature. Slices of liver were pushed through a wet screen (80-mesh) under a constant stream of α-MEM and the mixture allowed to stand for 5 min. The top two thirds of the cell suspension were removed and pelleted by centrifugation at 500 rpm for 10 min. The pellets were resuspended in fresh heparinised α-MEM containing goat serum and diluted to the appropriate cell concentration.

A total of fourteen pregnant does were used, seven of which were carrying twins. Recipient fetuses were exposed by maternal laparotomy and hysterotomy. The fetus was partially delivered through the hysterotomy incision taking care not to rupture the amniotic vesicle and the fetal sex determined by visual examination. BMT was performed by intraperitoneal injection of $9-24 \times 10^6$ cells from a sex-mismatched donor ($2-6 \times 10^8$ cells/kg estimated body weight) into 60–79-day recipient fetuses using a 22-guage needle. Fetal liver cell transplantation (FLT) was performed by i.p. injection of 15×10^6 male cells into 60-day recipient female fetuses (4×10^8 cells/kg estimated body weight) using the same protocol. The total volume administered in either procedure was no greater than 1.0 ml. After infusion of the suspension, the fetus was returned to the uterine cavity and the myometrial and abdominal incisions closed. Eight transplants were performed using mature allogeneic bone marrow and four using fetal liver cell suspension. In the case of twin pregnancies only one fetus was transplanted. Two animals acted as experimental controls in which the surgical procedure but no injection was performed. Pregnancy was allowed to continue and supported during the peri-operative period with exogenous progesterone administered i.m.

Peripheral blood samples were obtained for karyotyping within two weeks of birth from all surviving kids. In a control study, chromosome analysis was also performed on blood specimens from male and female donors mixed in measured amounts without knowledge of the source of the samples. The buffy coat was removed aseptically from heparinised blood samples after spinning for 45 min. About 5×10^6 mononuclear cells were added to 5 ml of culture medium (α-MEM with 25 mM Hepes containing 20% fetal calf serum, 1% glutamine, 3% pokeweed mitogen and 1% penicillin/streptomycin). After 72 h of culture at 37 °C, 0.03 ml colcemid (Gibco, NY, USA) was added to arrest the cell at the metaphase stage. 2 h later cultures were centrifuged for 10 min at 1000 rpm and the pelleted cells resuspended in hypotonic (0.067 M) KCl to a final volume of 8.0 ml and incubated for 22 min at room temperature. After being washed three times with a fixative of 3:1 methanol:acetic acid, aliquots of the suspension were dropped on slides, air-dried and stained with 10% Giemsa stain for karyotyping. The number of cells counted was dependent on mitotic activity, but for samples listed in table 1 they varied from 52 to 91 cells.

Results and discussion. Seven out of the total of 14 pregnancies ended in spontaneous abortion, two of which had carried twins and included both does acting as experimental controls. One pregnancy ended in fetal resorption and stillbirth of the twin. One doe carrying twins died during the post-operative period. Eight animals were born at term,

Table 1. Number of XY cells (donor) and XX cells (recipient) in goat kids injected at 60 days of gestation with fetal liver cells

Animal number	Phenotypic sex	Cell karyotype*	Number of cells counted (%) Postnatal age		
			14 days	98 days	231 days
202	F	XY	1 (1.2)	1 (1.8)	1 (1.7)
		XX	84 (98.8)	54 (98.2)	58 (98.3)
202B	F	XY	2 (2.2)	0 (0)	1 (1.4)
		XX	89 (97.8)	52 (100)	72 (98.6)

* Cell karyotypes were determined from studies on peripheral blood lymphocytes.

Table 2. Number of XX and XY cells in control studies of mixed female and male blood

% Mix F:M	Number of cells counted (actual %)* XX	XY
0:100	0 (0.0)	200 (100.0)
50:50	61 (50.0)	61 (50.0)
90:10	196 (98.0)	4 (2.0)
90:10	59 (100.0)	0 (0.0)
90:10	56 (96.6)	2 (3.4)
90:10	82 (100.0)	0 (0.0)
90:10	56 (96.6)	2 (3.4)

* Cell karyotypes were determined from studies on peripheral blood lymphocytes.

three of which had been recipients of mature allogeneic bone marrow and two had received fetal liver cell suspension. Aborted fetuses were not available for post mortem examination. Chromosome analysis performed on surviving FLT recipients showed that both animals (No. 202 and No. 203 B) were chimeric at 14 days of age. One of these animals (No. 202) was the product of a single pregnancy and the other (No. 203 B) had a female twin. This excluded the possibility that male cells were derived in utero from a twin of the opposite sex. Furthermore, these animals maintained chimerism for 7 months after birth without clinical evidence of graft-versus-host disease (GVHD) (table 1). In contrast, chimerism was not demonstrated in any recipients of mature allogeneic bone marrow. The results of control karyotyping studies performed on mixed blood samples indicated greater discrepancy between theoretical and actual percentages reported when the amount of donor blood added was low (table 2).

As with all surgical procedures on the early gestational fetus, there was a high incidence of spontaneous abortion (50%), but this observation is similar to that of other investigators [3,4]. Previous work [5] suggests that the incidence of postoperative abortion in the goat is higher than in the sheep and is probably due to regression of the corpus luteum caused by release of prostaglandin $F_{2\alpha}$ from the caprine uterus during surgery [6].

Hemopoietic chimerism is well documented in mammals whether this has occurred naturally [7,8] or has been induced experimentally [3,4,9] and is dependent upon tolerance of allogeneic cells by the fetal immune system. The pioneering investigations by Silverstein et al. [10] on skin allograft tolerance in fetal sheep have shown that grafts performed prior to day 77 of gestation were not rejected. In experiments originally reported by Zanjani et al. [4], successful engraftment was induced in sheep without GVHD, by performing intrauterine allogeneic BMT during late gestation (85–105 days). These data suggested that in the adult-to-fetus transplantation, tolerance of the donor cells persisted later in prenatal development than might be predicted on the basis of Silverstein's results. However, Zanjani and colleagues recently reported development of GVHD in these animals and long-term hemopoietic chimerism without GVHD was only achieved using fetal liver cells as the source of hemopoietic tissue [3]. The successful induction of hemopoietic chimerism in the goat fetus at this stage of gestation is further evidence for the physiological similarity between sheep and goats. Although the percentage of donor cell engraftment achieved in this report was low (1–2%), the results of control studies indicate this is a conservative estimate and engraftment could be as high as 10%. Failure to detect hemopoietic chimerism in recipients of allogeneic BMT may reflect the insensitivity of the technique used to determine engraftment, or alternatively, the developing hemopoietic tissues in fetuses at this stage in gestation may not allow for colonization by stem cells derived from mature hemopoietic tissue.

The overall significance of studies using animal models in the evaluation of allogeneic BMT as a means of enzyme replacement for human LSD, has been ambiguous for two reasons. Firstly, immunological complications of post-natal transplantation (graft rejection and GVHD) require mandatory immunosuppression of the recipient. Secondly, many of these studies have been performed at a time of advanced disease and therefore assessment occurs during a period in which amelioration of clinical signs and biochemical observations may be difficult to appreciate. Transplantation of normal fetal liver cells into preimmunocompetent goat fetuses affected with β-D-mannosidosis may provide an alternative strategy for evaluation of hemopoietic stem cell transplantation in the treatment of human LSD. Results of these studies confirm the feasibility of this approach, though future studies would be improved by the development of a more sensitive and accurate technique for detection and quantitation of the extent of donor tissue engraftment.

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