Rejection patterns in allogeneic uterus transplantation in the mouse

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BACKGROUND: Transplantation of the uterus in the mouse has been developed as a model system for research towards human uterine transplantation. Previous studies in a mouse model have demonstrated that a syngeneic uterus transplant can give rise to normal offspring. The aim of this study was to characterize the time course of rejection in a fully allogeneic mouse uterus transplantation model. METHODS: Uteri of BALB/c mice were transplanted to a heterotopic position in C57BL/6 recipients, whose native uteri were left in situ. The blood flow of the uteri, their gross appearance and general histology and the density of T-lymphocytes were examined on postoperative days 2–28. RESULTS: Macroscopic signs of rejection were apparent from day 5. At the light microscopy level, minimal inflammatory changes were seen from day 5 and massive inflammation was seen from day 10 to day 15. At day 28, necrosis and fibrosis were seen. The density of T-lymphocytes (CD3+) was increased in the grafted uterus from day 2 in the myometrium and from day 5 in the endometrium. Blood flow in the grafted uterus was reduced from day 15. CONCLUSION: A murine model to study rejection of allogeneic uterus transplants was characterized. Signs of rejection were seen from day 2 to day 5 and severe rejection was seen from day 10 to day 15. The data will be useful in future studies on immunosuppressants in this model.

Key words: allogeneic/mouse/rejection/transplantation/uterus

Introduction

Uterine transplantation is currently being developed as a potential treatment for patients with absolute uterine factor infertility (Altchek 2003; Brännström et al., 2003a, b). Patients who could be considered for this procedure are women who have preserved ovaries but with absence of the uterus due to either a congenital Müllerian anomaly or previous hysterectomy. Additionally, patients with a preserved but non-functioning uterus in regard to implantation of an embryo or the maintenance of pregnancy (due, for example, to leiomyoma or intrauterine adhesions) may be considered for this procedure (Brännström et al., 2003b). A case of a human uterus transplant was recently reported in a woman who had previously lost her uterus at peripartum hysterectomy (Fageeh et al., 2002). This transplanted uterus survived only for 99 days despite initial immunosuppression with cyclosporin A, azathioprine and prednisolone, subsequently boosted by antithymocyte globulin. Thus, we feel that the attempt was premature since important issues, such as the mechanisms of uterine rejection, suitable immunosuppressants and the tolerability of the transplanted uterus in regard to pregnancy, had not been tested in any animal model.

When a new type of organ is considered for transplantation, the surgical technique, the process of rejection of that particular organ and the means of suppressing rejection have to be thoroughly investigated. The mechanism of rejection of the uterus has not been particularly investigated, although allogeneic transplantation was reported in the dog (Yonemoto et al., 1969; Scott et al., 1970; Wingate et al., 1970; Paldi et al., 1975), rabbit (Confino et al., 1986) and rhesus monkey (Scott et al., 1971) some decades ago. These reports merely concluded that rejection occurred and they did not characterize the process in any detail. Recently, data have been presented on methods for en bloc uterus–oviduct–ovarian transplantation in the rat, and it was stated that severe rejection occurred within 2 days (Lee et al., 1995; Jiga et al., 2003; Motoc et al., 2003). Our group has chosen to develop a uterine transplantation model in the mouse (Racho El-Akouri et al., 2002) since this species has apparent advantages over other animal models in this research because of its well-characterized reproductive physiology and immunology and the ready availability of inbred and gene-deleted mouse strains. In the syngeneic mouse uterine transplantation model, normal rates of implantation and pregnancy are seen (Racho El-Akouri et al., 2003).

Knowledge about the mechanisms of organ specificity with respect to the intensity and type of rejection is increasing. The uterus, with its special local immunology during pregnancy, when the semiallogeneic fetus survives despite the presence of maternal...
T cells specific for paternally inherited histocompatibility antigens, may have unique features. Since the uterus may have special immunological properties also with respect to local mechanisms of rejection, it is important to study the rejection process in detail in this organ and later on to develop ways to control rejection or to induce tolerance, in both the pregnant and the non-pregnant state. Immune-mediated graft rejection would be the principal challenge in uterine transplantation, as in the transplantation of other organs.

In the present study, the rejection pattern of transplanted uteri in a fully allogeneic mouse model was characterized by several methods.

**Material and methods**

**Animals**

Inbred female C57BL/6 and BALB/c (M&B, Ry, Denmark) mice 6–8 weeks of age were housed in controlled conditions (21–23°C, relative humidity 50–60%, illumination between 07.00 and 19.00 h) with water and pelleted food *ad libitum*. Thirty-two out of a total of 63 operated recipient mice were excluded from the study due to immediate postoperative complications. These 32 animals were lost, either because of haemorrhage (*n* = 11) or because the transplanted uteri did not show any blood flow through the uterine artery and/or vein (*n* = 21) at immediate inspection after vascular anastomosis had been performed. The experiments were approved by the local animal ethics committee and were carried out according to the principles and procedures outlined in the Guide for the care and use of laboratory animals (National Institutes of Health, USA).

**Donor operation**

BALB/c mice served as uterus donors. Isolation of one uterine horn, including the cervix and connecting vasculature, was done as previously described (Racho El-Akouri et al., 2002, 2003). The reason for using a unilateral uterine horn rather than a complete uterus was that the unilateral procedure enabled isolation of the pelvic vasculature on the dorsal aspect of the uterus (Racho El-Akouri et al., 2002). Briefly, under isoflurane (2%) anaesthesia, the right uterine horn and the cervix together with the feeding and draining vessels (right uterine vessels, right hypogastric vessels, right common iliac vessels, vena cava and aorta) were gently isolated by microsurgical dissection. Other branching vessels (right inferior mesenteric vessels, right inferior epigastric vessels, left common iliac vessels, caudal artery, right external iliac vessels, right pudendal vessels and lumbar vessels) were cuterized and cut to prevent leakage from the specimen. The bladder was removed and the cervix was transected below its attachment to the bladder. The aorta and vena cava were then separated from the level of the branching of the renal vessels and 3–4 mm caudally. A ligature was placed cranially to the tip of the right uterine horn and the uterus was divided from the oviduct and ovary and dissected free from the dorsal peritoneum. The aorta and the vena cava were tied off caudally to the branching of the ovarian vessels and, after cannulation of the aorta (30 G needle) and incision into the vena cava, the specimen was gently flushed with 0.154 mol/l NaCl (4°C) supplemented with 100 IU/ml heparin sulphate (Leo Pharma, Malmö, Sweden) and 0.2 mg/ml xylocaine (Astra Zeneca, Göteborg, Sweden) to remove blood from the uterus. The uterus was then kept in 0.154 mol/l NaCl (4°C) for 20 min during surgical preparation of the recipient animal (see below) and back table preparation of the specimen.

**Recipient operation**

C57BL/6 mice served as uterine graft recipients. Surgery (under isoflurane anaesthesia) was performed through a midline laparotomy incision after subcutaneous administration of 15 IU heparin sulphate. The aorta and vena cava were mobilized and haemostatic clamps were placed *en bloc* around the vessels. Aortic–aortic and caval–caval end-to-side-anastomoses were performed using interrupted and continuous (11–0 nylon) suture techniques, respectively. The cervix of the graft was then exteriorized through a circular incision on the right side of the abdomen and sutured to the skin to create a stoma. The laparotomy scar was closed and 1 ml of a solution containing 0.154 mol/l NaCl and glucose (50 mg/ml) was administered subcutaneously to adjust for fluid loss and to correct any hypoglycaemia.

**Gross morphology and laser Doppler flowmetry**

The mouse was anaesthetized and a midline laparotomy was performed. The uteri and stomas of the recipients were initially examined with the aid of an operating microscope at days 2, 5, 10, 15 and 28 after surgery, during anaesthesia. A laser Doppler flowmetry method (Öberg, 1990) was then used to estimate tissue blood perfusion. The method has previously been used for studies of blood flow in ovaries of the rat (Zackrisson et al., 2000) and mouse uterus (Racho El-Akouri et al., 2002) at specific times (see above). A laser Doppler miniprobe (Perimed, Järfälla, Sweden) was placed in the lumen of both the transplanted uterus and the native uterus to record blood flow (Periflux 5000 flowmeter; Perimed) for periods of 2–3 min. The blood flow was expressed as arbitrary perfusion units (PU).

**Light microscopy and immunohistochemistry**

The mice were killed by cervical dislocation during anaesthesia at specific times (see above). The native and grafted uteri were dissected out and bisected. One part of the bisected uterus was fixed in solution containing 4% w/v paraffomaldehyde in 0.1 mol/l sodium cacodylate buffer (pH 7.4). The tissue was then dehydrated, embedded in paraffin and sectioned (~4 μm) for light microscopy. Staining with haematoxylin and eosin, elastin–van Gieson and periodic acid–Schiff was performed. The slides were examined in a light microscope and digital images were obtained with a SPOT™ camera (Diagnostic Instruments, Sterling Heights, MI, USA).

The other half of the uterus was frozen in OCT (Sakura Finetek Europe, Zeterwoude, The Netherlands). This uterine tissue (native, graft) of postoperative days 2, 5 and 10 was sectioned (~4 μm), fixed in acetone for 5 min and then blocked in 1.5% normal rabbit serum in phosphate-buffered saline (PBS; NaCl 0.13 mol/l, Na2HPO4 0.015 mol/l, KH2PO4 0.004 mol/l) for 30 min. The sections were incubated at room temperature for 1 h with the primary monoclonal antibody (rat anti-mouse CD-3 molecular complex; BD PharMingen, San Diego, CA, USA) at 1:1000 dilution in PBS and 1.5% normal rabbit serum. After three washes in PBS, the sections were incubated in peroxidase-conjugated, biotinylated, mouse-adsorbed, rabbit anti-rat IgG (Vector Laboratories, Burlingame, CA, USA) in PBS and Vectastain ABC reagent (Vector Laboratories) for 30 min, followed by incubation with the peroxidase-substrate diaminobenzidine tetrahydrochloride for 5 min. The sections were counterstained with haematoxylin. Negative controls were performed by replacing the primary antibody with PBS.

The number of positive cells was estimated by counting the number of stained cells within a lined grid (10 × 10 squares) occupying an area on the section of 0.125 mm2 using a 20× objective and 10× eyepiece. In each uterus, five randomly chosen areas were counted within the myometrium and five areas were counted within the endometrium (defined as the region from the luminal surface to the most basal glandular epithelium). The mean of these values was calculated and this value was used as a data point. One section from the native and one section from the grafted uterus from each of the five animals in each group were counted. Counting was performed independently by two
of both the glandular and the luminal epithelium. Blood congestion in small vessels (mainly venules) was also observed (Figure 2c). However, there was no evidence of endarteritis in the arteries or of endothelialitis in the larger veins.

At days 10 and 15, the typical features overlapped somewhat between the animals of the two groups. At day 10, the uteri were heavily inflamed throughout the uterine wall and numerous lymphocytes were present in the glandular epithelium (Figure 2d, g). Focal apoptosis was also observed, but necrosis was not apparent at this time point. The blood vessels were congested and there was evidence of endarteritis in larger arteries, with lymphocytes invading the endothelial layer into the intima (Figure 2b). At day 15, four out of five of uterine transplants showed histological signs of severe rejection, with arterial thrombi and necrotic parenchyma (Figure 2f). One transplant of day 15 had a histological appearance similar to those of day 10. The native uteri of day 15 (Figure 2e) appeared slightly oedematous but without any visible increase in inflammatory cells. At day 28, no viable parenchyma was present in any of the transplanted uteri. There was massive central necrosis and only a peripheral rim of fibrotic tissue.

The density of T-cells in the myometrium (Figure 3a) and the endometrium (Figure 3b) was higher in the grafted uteri (Figure 2i) compared with the native uteri (Figure 2j) from day 2 and day 5, respectively. There was no time-dependent change in the T-cell density in the myometrium or endometrium in the native uterus (Figure 3a, b). However, the density of T cells in both the myometrium and endometrium in the grafted uteri was significantly higher at days 5 and 10 compared with day 2 (Figure 3a, b).

**Blood flow**

The blood flow was significantly lower in the grafted uteri compared with the native uteri at all time points (Figure 4). There was no significant time-dependent change in the blood flow of the native uteri. In the grafted uteri there was a significant change in blood flow, with lower levels at days 15 and 28 compared with days 2 and 5.

**Discussion**

To achieve a suitable model system for basic studies on the issue of uterine transplantation, we have developed a mouse model using microsurgical vascular anastomosis and heterotopic placement of the grafted uterus parallel to the native

<p>| Table 1. Summary of results regarding gross appearance of transplanted uterus |
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*Swelling is defined as an outer uterine diameter more than double that of the native uterus.

*Marked shrinkage is defined as stoma opening less than half of the stoma at day 0.
Rejection of uterus transplants (Racho El-Akouri et al., 2002). In the latter study, the surgical technique was developed and established using inbred mice (B6 CBAF1) as both donor and recipient, and the transplanted uterus showed the capacity to harbour pregnancies (Racho El-Akouri et al., 2002) and to produce offspring with a normal postnatal growth trajectory (Racho El-Akouri et al., 2003). Collectively, these initial studies demonstrated the feasibility of this model in research to develop uterine transplantation as a clinical procedure for the treatment of uterine infertility. In the present study, we characterized the rejection mechanisms by making observations at several levels in a fully allogeneic uterine transplantation model in mice.

A fully allogeneic mouse model with mice of the BALB/c strain as uterus donors and mice of the C57BL/6 strain as recipients was used. The reason for using C57BL/6 mice as recipients was that this strain is the background strain used in the development of most knockout and transgenic mice. We anticipate that these knockout or transgenic mice will be used in future research designed to investigate in more detail the mechanisms of rejection in a transplanted uterus or in experiments on the physiology of pregnancy. Moreover, our previous experiments involving syngeneic transplantation were performed in F1 hybrids of the recipient strain of the present study, C57BL/6, and CBA mice (Racho El-Akouri et al., 2003). The donor mouse strain (BALB/c) used in the present study is the albino mouse strain which is most widely used in biomedical research (Potter, 1985).

The major histocompatibility (MHC) complex of the mouse, H-2, has its genetic loci on chromosome 17 and is homologous to HLA in the human. Haplotypes of the H-2 complex are determined by combination of alleles of the class I, class II and class III genes (Klein et al., 1983). The H-2 haplotypes of BALB/c and C57BL/6 mice are termed H-2<sup>a</sup> and H-2<sup>b</sup> respectively. These two haplotypes show dissimilarity in all alleles except one (the Q1 locus of class Ib). Thus, the almost fully
The mouse model for organ transplantation has become increasingly popular with advances in microsurgery and molecular biology. However, knowledge about the rejection patterns in these murine models is limited. Experiments involving heart, kidney, liver and intestinal transplantation between different mouse strains have evaluated C57BL/6 mice as donors and BALB/c mice as recipients (Zhang et al., 1996). This is the opposite situation to the present study, and no evaluation was performed concerning BALB/c as donor and C57BL/6 as recipient. In the study of Zhang and colleagues, spontaneous acceptance was seen in 72 and 20% of liver and kidney allografts respectively. However, the heart and intestine allografts were rejected after around 9 days. The results were similar when BALB/c were used as donors to CBA mice. In a study using orthotopic transplantation of carotid allografts to test the effects of immunosuppressive drugs, C57BL/6 and BALB/c mice were tested both as donors and recipients (Matsumoto et al., 2004). It was shown that rejection by C57BL/6 recipients was not suppressed by immunosuppressant drugs as much as when BALB/c were recipients. Taken together, these data indicate that an organ such as the heart, which is similar to the uterus in its large content of muscular tissue, would be rejected within a shorter time than 9 days when BALB/c donors and C57BL/6 recipients were used. This time of rejection is somewhat shorter than what was found in the present study concerning the uterus.

In the present study, a fairly large proportion of animals had to be excluded due to immediate postoperative complications. This is most likely a consequence of the technically very difficult dissection and microsurgery involved in this procedure. The failures were most likely not attributable to the so-called immunologically mediated hyperacute rejection, since a similar portion of mice with unsuccessful grafts was seen in our previous study using a syngeneic model (Racho El-Akouri et al., 2003). During the study it was found that the manipulation of the graft and the warm ischaemic time had to be reduced to a minimum to prevent these complications.

The macroscopic appearance of the transplanted uterus of the present study was recorded with respect to both the cervical mucosa (stomal end) and the intra-abdominal serosal surface appearance. The first obvious signs of rejection were swelling and a colour change, which was seen only by intra-abdominal appearance at day 5. This time corresponds with the first...
histological signs of inflammation. These results of early macro-
scopio signs of rejection are comparable to what has been
reported previously in rat cardiac allografts, with oedema seen
around day 4 (Hayashi et al., 1991). Similarly, uterine allo-
grafts in the dog were swollen and hyperaemic at days 4–5
(Wingate et al., 1970).

The macroscopic changes which were observed in the stoma
of the uterine allograft correlated fairly well with the macro-
scopic intra-abdominal appearance as well as with the histologi-
signs of rejection. The first macroscopic sign of rejection
seen in the stoma was on day 10, which was at a stage when
marked increases were seen in the density of T cells in both the
endometrium and myometrium. Thus, the exteriorized cervical
end of the uterus can be used conveniently to assess the viabil-
ity of the organ and the different phases of rejection of the
transplants. A similar situation is present in the newly
developed mouse model for small bowel transplantsations (He
et al., 1998; Dindelegan et al., 2003), with a similar cutaneous
stoma. The stoma would also allow a non-invasive means of
obtaining biopsies from the organ for more detailed assessment
of rejection by histology or immunohistochernistry.

The rejection of the grafted uterus became macroscopically
more evident with time when the uterus became more blackish in
colour, with a fully abnormal texture. The stomas became
darker in colour and shrank. In the tissue sections, increased
infiltration of inflammatory cells, endarteritis and arterial
thrombi with necrotic parenchyma were seen at days 10 and 15.
These results correspond to severe acute rejection and are simi-
lar to those of cardiac allografts at days 8–15 (Zhang et al.,
1996; Riederer et al., 2002). The allograft survival and severity
of rejection is not only dependent on the MHC mismatch but is
also dependent on the specific organ that is transplanted. Thus,
spontaneous acceptance of transplanted liver and kidneys is
seen in the mouse (Zhang et al., 1996). From this point of view
it seems as if the heart and the uterus are similar, with no spon-
taneous acceptance and a similar timing of the rejection phases.

The endometrium is part of the common mucosal immune
system that has special features to allow immunological toler-
ance of semen and conception while actively defending the
uterus from pathogenic bacteria and viruses. The histological
examination in the present study indicated that rejection of
the endometrium is more rapid than that of the myometrium. Thus,
early lymphocyte infiltration was seen in the endometrial
glands, whereas lymphocyte infiltration occurred later in the
myometrium. The myometrium appeared to have the greatest
resistance to rejection and retained its tissue structure for the
longest time. These results are comparable to the results of a
study of allotransplanted uteri performed in the dog (Scott
et al., 1970), in which the grafted uterus showed total necrosis
of the endometrium but only a mononuclear infiltration and
marked oedema of the myometrium 2 weeks after trans-
plantation. The mechanism behind the early rejection of
the endometrium is most likely an early invasion of leucocytes,
as illustrated by the increase in T lymphocytes in the endometrium
already at day 2.

In the present study, reduced uterine tissue blood flow was
seen in the grafted uteri compared with the native uteri at all time
points. In our previous study in the syngeneic mouse model
(Racho El-Akouri et al., 2002), we were not able to demonstrate
any difference in blood flow when assessed 2 and 4 weeks post-
operatively. It may well be that the blood flow in those syngeneic
grafts was lower during the first weeks after transplantation and
that the blood flow then increased to levels similar to that of the
native uterus. An explanation of this normalization of blood flow
in the syngeneic graft may be that the acutely decreased blood
flow, due to the absence of blood flow in the uterine branches of
the ovarian artery, is slowly compensated for by blood flow from
the uterine artery. In the allogeneic model of the present study,
early rejection events will prevent the normalization of blood
flow and eventually lead to a decrease in blood flow. However, in
small bowel transplants in a mouse model no decrease in blood
flow was seen after syngeneic transplantation when evaluated
from day 1 to day 8, but a decrease was seen in allogeneic trans-
plants from day 3 (Dindelegan et al., 2003).

The histological correlates to this decrease in uterus blood
flow is naturally the major perivascular changes which were
seen from around day 15. Furthermore, thrombosis was seen in
the rejected grafts after allotransplantation of the uterus in the
dog (Wingate et al., 1970). In that study, thrombosis was
mainly seen in the large and medium-sized arteries and veins
from day 3 to day 28 after transplantation.

In conclusion, uterine allotransplants show early signs of
rejection from day 5 and severe rejection from day 10 to day 15
after transplantation. The rejection pattern of this murine
model is fairly uniform, in contrast to several murine strain
combinations of kidney transplantsations, which showed both
spontaneous acceptance and rejection (Zhang et al., 1996). The
rejection pattern of the uterus in a murine model seems to be
most similar to that of heart transplants.

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