Morphologic and Functional Study of Heterotopic Splenic Tissue Allografts in Rabbits

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Abstract

Objectives: To assess the viability and induction of immunotolerance of nonvascularized splenic alloimplants. The phagocytic functions of splenic implants also were studied.

Materials and Methods: Thirty-six adult female New Zealand and California rabbits were used, and these animals were divided into the following 5 groups: (n = 6 / groups 1-4) group 1 (sham operations); group 2 (total splenectomy); group 3 (implantation of autologous sliced splenic tissue in the greater omentum following splenectomy); group 4 (implantation of allogenic sliced splenic tissue in the greater omentum after splenectomy); and group 5 (n = 12) (implanting allogenic sliced splenic tissue in the greater omentum after splenectomy and receiving oral cyclosporine at a dosage of 40 mg/kg/d). All animals were followed for 120 days after the operations, then received venous injections of China ink (groups 1, 2, 3, 4, 5A) or a colloidal radiopharmaceutical (group 5B), and subsequently underwent reoperations. Hematimetric examinations were performed, and the histologic aspects and phagocytic functions of the implants were assessed.

Results: Spontaneous immunotolerance was not induced by sliced splenic allografts implanted in the greater omentum. The use of cyclosporine did not preserve the viabilities of the implants. All animals in group 3, which were subjected to autologous implants, exhibited viable implants that exhibited phagocytic function, although this phagocytic function was reduced compared with that of the normal spleen.

Conclusions: No viable spleen alloimplants were observed regardless of the presence of cyclosporine. Spontaneous immunotolerance was not induced by sliced splenic alloimplants.

Key words: Transplant, Homologous, Spleen, Immunotolerance

Introduction

Post-splenectomy complications are described in the literature and are primarily related to infection and thromboembolic events.1 Spleen conserving techniques have been developed and include nonoperatory treatment of splenic trauma, partial resections, and operations for auto-implants or allografts.2-4 Splenic regeneration in the peritoneum was observed in splenectomized dogs as early as the nineteenth century.5 The term splenosis was suggested by Bubchbinder and Lipkoff based on cases of implants of splenic tissue in the abdominal cavity after trauma.6 The splenic implants regenerate after initial necrosis and take the form of nodules with capsules and white and red pulps. The implants also exhibit vascularization similar to that of the entire organ.7,8 Many sites were used for splenic implantation, including the greater omentum, subcutaneous sites, mesenteric sites, the liver (through the portal vein), muscle, and preperitoneal, and retroperitoneal sites. The data suggest that splenic implants achieve better function when they
are located in sites with drainage to the portal vein.9-11 There is evidence that implants in young animals regenerate better than those in adults.7,9 Studies that had evaluated the induction of immunotolerance show that the spleen is capable of inducing immunotolerance after transplant in rodents. In 1974, Bitter-Suermann’s work described the spontaneous viability of splenic allografts in rats, even those that were incompatible in terms of the major histocompatibility complex.15 According to Suzuki and associates, the spleen can induce immunotolerance after organ transplant in rats, particularly cardiac transplants.16

Cellular chimerism is verified when the peripheral blood and donor cells are found in the thymus, bone marrow, and lymph nodes after viable splenic transplants, and allows for the conclusion that dynamic interactions between the immune cells of the donor and recipient are occurring. When the splenic graft is lost, this chimerism disappears.17 The transplant of splenocytes with bone marrow can establish allogeneic chimerism and induce immunotolerance in mice.18,19 However, Gollackner and associates noted the lack of immunotolerance induction when splenic cells are transplanted rather than the entire organ.20

Cyclosporine is an effective immunosuppressive drug used for transplants. Studies conducted in the same research field of this work have used cyclosporine in rabbits that have undergone skin, heart, and ovary transplants.21-23

Our objective was to assess the viability and the induction of immunotolerance of nonvascularized splenic alloimplants. The phagocytic functions of splenic implants also were studied.

Materials and Methods

This work was conducted according the recommendations of the International standards of animals protection, the Brazilian code of animal experimentation, the ethics committee in animal experimentation of Federal University of Minas Gerais (UFMG-CETEA), and was approved under protocol number 94/2006.

Thirty-six adult female rabbits from New Zealand and California breeds were randomly allocated into the following 5 groups:

Group 1 (n = 6), control-sham operation: 3 New Zealand and 3 California rabbits were given a laparotomy and spleen manipulation, followed by laparorrhaphy.

Group 2 (n = 6), asplenic control: 3 New Zealand and 3 California rabbits were given a total splenectomy.

Group 3 (n = 6), autogenic implants: 3 New Zealand and 3 California rabbits were given a total splenectomy followed by autogenic implantation of sliced splenic tissue in the greater omentum.

Group 4 (n = 6), allogenic implants without immunosuppression: 3 New Zealand and 3 California rabbits were given a total splenectomy followed by crossed transplant of sliced splenic tissue into the greater omentum. Cyclosporine was administered at a dosage of 40 mg/kg/day. Phagocytic function was evaluated via 2 methods:

Group 5A (n = 6), phagocytic function was evaluated via colloid carbon.

Group 5B (n = 6), phagocytic function was evaluated via scintigraphic study with 99m-phytate-technetium.

Blood samples were obtained from all the animals before the operations via puncture of the central artery. Complete blood counts were performed. After 120 days, when the animals were reoperated on and killed, new complete blood counts were performed, and the blood samples were obtained with the same technique described above.

To evaluate the phagocytic activities of the spleen and liver, China ink was injected 120 days after the first operation. The China ink composition includes colloidal carbon particles measuring 2 to 10 μm. The ink was prepared in a 50 g/100 mL watery dilution. The injections of China ink were performed via cannulation of the veins of the rabbits’ ears using disposable 5-mL plastic syringes and disposable 13 × 4-mm needles. The China ink was injected in volumes that corresponded to 1 mL per 1000 g of the rabbit’s weight. The phagocytic function was evaluated qualitatively by counting clusters of tiny carbon particles inside the macrophages.

The radiopharmaceutical used in the scintigraphic studies was 99m-phytate-technetium. The radiation dose administered was 110 megabecquerel (MBq), which corresponds to 3 millicuries (mCi). The radio-pharmaceutical injections were performed in veins in
the rabbits’ ears. Twenty minutes after the injections, the scintigraphic studies were performed in a gamma camera device.

The animals were anesthetized with ketamine (80 mg/kg) and xylazine (15 mg/kg) via an intramuscular injection into the gluteus. A 6-cm laparotomy beginning at the xiphoid process and continuing in the cephalocaudal direction was made in each animal. Group 1 underwent sham operations in which only the splenic manipulation and laparorrhaphy were performed. Following the exploration of abdominal cavity, the musculo-aponeurotic layer was closed with a running suture of 2-0 silk. The skin was sutured with separated stitches of 3-0 monofilament nylon.

In group 2, the spleen was positioned to expose the blood vessels. After dissection, the splenic artery, splenic vein, and splenogastric vessels were ligated and cut, and the splenectomy was performed. After exploration of the abdominal cavity, the abdominal wall was sutured as in group 1. In group 3, in addition to the procedures described for group 2, the spleen was sectioned into slices of 0.5 cm × 0.5 cm × 0.3 cm and immediately sutured over the greater omentum of the animal (ie, heterotopic autogenic splenic implant) via separated stitches with 4-0 catgut. After reviewing the cavity, the abdomen was closed as described for group 1.

One hundred twenty days after the first operation, the animals were anesthetized again with ketamine (80 mg/kg) and xylazine (15 mg/kg) via intramuscular injection. Six hours undergoing the reoperations, the animals from groups 1, 2, 3, 4, and 5A received intravenous injections of China ink. After again opening and exploring the abdominal cavity, the greater omentum (ie, the site of splenic implants) was resected, and additional liver samples were collected. In group 1, splenic fragments also were collected.

In group 5B, the animals were subjected scintigraphic study before being reoperated on. The surgical procedure was identical to that described for the other groups. Next, the animals were killed via anesthesia by injecting 10 mL of 10% potassium chloride in the caudal vein.

Fragments from the region in the greater omentum at which the splenic tissue were implanted from the liver and from spleen itself and were fixed in 10% formalin. The samples were fixed on glass slides and submitted to 2 types of histologic staining: hematoxylin-eosin and eosin alone. The macrophages containing China ink were counted in 5 consecutive fields at ×400 magnification. The initial field was randomly chosen. The mean numbers of macrophages with colloids in their interiors were calculated.

Data are presented as the mean and the standard error of the mean. Wilcoxon tests were used to compare the pre- and postoperative values of each group. The Kruskal-Wallis test was used to verify differences in the various measures among the 5 groups. The mean numbers of macrophages containing colloidal carbon in the splenic tissue were calculated for group 1 (control) and for the implants in the greater omentum of the animals in group 3 (ie, the auto-implants). The Wilcoxon test was used to compare groups 1 and 3. The results were considered significant when $P < .05$.

Results

All animals tolerated the anesthesia and quickly recovered normal motor activity after the procedures.

In group 1 (control), histologic evaluation of the splenic fragments, greater omentum, and liver revealed normal histologies in these organs in all animals. The splenic and liver fragments exhibited phagocytosis of the colloidal carbon.

In group 2 (asplenic control), the livers were normal and exhibited darkened coloration due to capture of the nankin ink. The greater omentum appeared normal in all animals. Histologic evaluations of the livers revealed normal organ structures and points of colloidal carbon capture.

In group 3 (autogenic implants), splenic implants were found in the greater omentum of all animals. Macroscopically, the implants exhibited the appearance of small spleens. Histologic evaluation revealed splenic tissue within the fatty tissue of the greater omentum with strands of lymphoid cells containing capillary vessels and fibrosis. The splenic tissue exhibited areas of colloidal carbon capture that confirmed the hypothesis of phagocytic activity in the autogenous implants. The liver tissue was normal and exhibited capture of the colloidal carbon.
In group 4 (allogeneic implants without immunosuppression), adhesions between the organs and the inflammatory mass in the greater omentum were observed. Palpation of the greater omentum revealed nodules that corresponded to the sites splenic tissue implantation. After cutting the greater omentum, encapsulated whitish necrotic material was found. In other areas of the greater omentum, only fibrotic tissues were found. Histologic evaluation revealed areas of necrosis within fibrotic and fatty tissues. In 2 rabbits from the group, the omentum had small areas with remnants of partially necrotic lymphoid tissue that were free of colloidal carbon capture. The liver was normal, and the presence of the colloidal carbon in this organ indicated phagocytic activity.

In group 5A (allogenic implants and immunosuppression), exploration of the abdominal cavity revealed adhesions between the organs and the inflammatory mass in the greater omentum. Examination of the omentum revealed findings identical to those described for group 4 and no signs of viable implants. Histologic evaluations revealed areas of necrosis, fibrosis, and remnants of lymphoid tissue, and no colloidal carbon capture.

In group 5B (allogenic implants with immunosuppression and scintigraphic evaluation), scintigraphic evaluations of the living animals did not show capture of the nankin ink in the greater omentum. Only hepatic capture was observed (Figure 1). Macroscopic examination revealed alterations that were identical to those described for groups 4 and 5A. Histologic examination did not reveal viable implants in the omentum, and this finding is similar to that of groups 4 and 5A.

The results of the preoperative and 120-day postoperative complete blood counts revealed differences in the lymphocyte and segmented neutrophil counts of groups 4 and 5. Among these differences, 2 patterns were notable:

- An increase in the lymphocytes counts of group 4 after 120 days;
- A decrease in the percentage of lymphocytes in group 5 (5A and 5B) that was accompanied by a concomitant increase in segmented neutrophils after the period of 120 days. Table 1 shows the comparisons of the preoperative and 120-day postoperative values.

![Figure 1. Scintigraphic Study Using Technetium-99m Phytate](image)

Hepatic capture of the Technetium-99m Phytate. No capture was exhibited by the implants.

![Figure 2. Comparison of Preoperative and 120-Days Postoperative Percentages of Lymphocytes Between Groups](image)

Table 1. Comparison of the Preoperative and 120-Day Postoperative Hemogram Test Values With P Values (Wilcoxon Tests)

<table>
<thead>
<tr>
<th>Groups</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5A</th>
<th>5B</th>
</tr>
</thead>
<tbody>
<tr>
<td>P Value</td>
<td>.674</td>
<td>.223</td>
<td>.917</td>
<td>.753</td>
<td>.528</td>
<td>.138</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>.674</td>
<td>.223</td>
<td>.917</td>
<td>.753</td>
<td>.528</td>
<td>.138</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>.463</td>
<td>.249</td>
<td>.753</td>
<td>.833</td>
<td>.249</td>
<td>.462</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>3.45</td>
<td>2.07</td>
<td>9.17</td>
<td>9.16</td>
<td>.116</td>
<td>.753</td>
</tr>
<tr>
<td>Platelets</td>
<td>3.45</td>
<td>6.00</td>
<td>6.00</td>
<td>5.99</td>
<td>.753</td>
<td>.463</td>
</tr>
<tr>
<td>Leukogram</td>
<td>7.53</td>
<td>.674</td>
<td>.753</td>
<td>.463</td>
<td>.075</td>
<td>.345</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>.684</td>
<td>10.000</td>
<td>.833</td>
<td>* .027</td>
<td>* .027</td>
<td>* .027</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>.680</td>
<td>.854</td>
<td>.684</td>
<td>.028</td>
<td>* .027</td>
<td>* .028</td>
</tr>
</tbody>
</table>

*Statistically significant.

Among-group comparisons did not reveal differences in either the pre- or 120-day postoperative red blood cell, hemoglobin, hematocrit, platelets, or leukocyte counts. Differential analyses of the total leucocyte counts revealed no differences between groups 1, 2, 3, and 4 but did reveal differences between group 5 and the other groups. Group 5 exhibited an increase in the percentage of segmented neutrophils at the end of the experiment and a decrease in the percentage of lymphocytes. Figure 2 shows this decrease in the percentage of lymphocytes in group 5 after 120 days.

Pre: $P = .777$.
120D: $P = .200$.
$1 = 2 = 3 = 4 > 5A = 5B$. 
These results indicate that the autogenic implants exhibited less phagocytic activity than does the normal spleen. The comparison of the mean number of macrophages that contained nankin revealed a significantly greater capture by the normal spleen (177.30 ± 8.60) than by the autoimplants (74.17 ± 4.70) (P = .028).

Discussion

This study is part of a splenic research program. The allogenic implants used here represented an attempt to develop splenic surgery to match the developments of other branches of research related to organ transplant. Studies using rabbits have shown viable heterotopic cardiac transplant with anastomosis of the heart to abdominal vessels and immunosuppression using cyclosporine and thalidomide. Another study reported an increase in the viability of skin grafts in rabbits after the combined use of cyclosporine and thalidomide. Sliced allogenic ovary implants (without vascular pedicles) have been transplanted in rabbits using cyclosporine for immunosuppression and resulted in the maintenance of reproductive and endocrine function.

The technique for splenic transplant was chosen based on previous studies. Other techniques for the implantation of splenic fragments exist. Inuma and associates concluded that the best site for the implantation of splenic tissue is the greater omentum. The implants should stay within the peritoneal cavity to allow for drainage to the portal vein. The technique of allogenic implantation was adapted from the technique for autogenic implantation.

The choices of colloidal carbon as the dye and the use of radiolabeled colloid to evaluate phagocytosis were based on previous studies. The colloidal carbon is clearly visible inside macrophages, particularly when of eosin or green light stains are used. These stains allow for the observation of cell contours and highlight carbon pigments inside the cells and enable the counting of the macrophages that contain pigments. Colloidal radiopharmaceuticals have been used in previous studies. In the present study, the choice of 99m-phytate-technetium was based on its use in clinical practice.

The results found in this study are similar to those previously published. Resende measured the same hematimetric values used in this study in patients who had undergone total splenectomy, subtotal splenectomy, or autogenic implants after splenectomy, and found no difference. Transient thrombocytosis has been described after splenectomy; however, the peak platelet count appears to occur between the seventh and twentieth days after the operation. Studies that have compared laboratory tests of renal transplant patients with those of healthy individuals have shown lymphopenia without alterations in the total leukocyte counts.

The autogenic implants were viable and exhibited phagocytic activities 3 months after the operations. These findings are similar to those of other studies. However, there is still debate regarding whether such implants exhibit the full range of function of the normal spleen. In the present study, the autogenic implants regenerated and preserved their phagocytic functions in all animals. The histologic evaluations revealed normal splenic architectures with reduced phagocytic function compared with the control group. Smith and associates found reduced phagocytic cell numbers and reduced phagocytic activity in implants compared with normal splenic tissue. Although it does not restore all splenic function, the maintenance of splenic tissue with autogenic implants is an option for preserving splenic tissue and some of its benefits.

In the groups that were subjected to splenectomy and allogenic implants of sliced splenic tissue, no viable implants were present after 3 months. Histologic and phagocytic evaluations did not reveal any splenic tissues at the sites of implantation. The presence of fibrotic and necrotic tissues indicates that the recipients rejected the implant, even when cyclosporine was used. The 3-month interval before evaluating the implants was based on studies of autogenic implants in which the implants regenerated after initial necrosis. In the present study, the allogenic sliced splenic implants were unable to induce spontaneous immunotolerance. The immunotolerance that has been observed in rats might not be reproducible in other animals. The differences between rodents and larger animals might be due to patterns of the expression of class II major histocompatibility complex antigens in the vascular grafts. These antigens are potent stimulators of T helper lymphocytes and are lacking in rat endothelial cells, but they are present in larger animals and humans.
Conclusions

No viable spleen alloimplants were observed, even in the presence of cyclosporine. Spontaneous immunotolerance was not induced by the sliced splenic alloimplants.

References

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