Segmental Difference in Epithelial and Mucosal Barrier Functions between the Jejunum and the Ileum in Cold Storage Small Bowel Grafts in the Rat

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Summary: The purpose of this study was to determine the differences in epithelial and mucosal barrier function between the jejunum and the ileum at transplantation. Rat small bowel was preserved in cold University of Wisconsin solution for 0, 12 or 24 hrs. Time-related segmental differences in the potential differences, at rest (rPD) and upon glucose stimulation (PDgs), and in the permeation rates of phenolsulfonphthalein (PSP) and polyethylene glycol 4000 (PEG) as non-absorbable markers were studied after a 20-min reoxygenation period following cold preservation. Time-related histologic damage during cold preservation was also evaluated. After 24 hrs of preservation, when the villi were denuded both in jejunum and ileum, the rPD (1.4±0.3 mV/cm²) and PDgs (1.4±0.3 mV/cm²) of the jejunum were significantly lower than those of the ileum (1.9±0.3 mV/cm² and 2.1±0.3 mV/cm², respectively). A difference in PEG permeation rates occurred after 24 hrs of preservation. The permeation rate of PSP in the jejunum was 10.5±1.0%, which was significantly higher than that in the ileum (8.6±1.0%) after 12 of preservation, when subepithelial edema occurred both in the jejunum and ileum. Our functional study demonstrated that, in the rat small bowel, the ileum is more resistant than the jejunum to cold preservation.

Key words small bowel transplantation, preservation, ischemia & reperfusion injury, potential difference, mucosal barrier function

INTRODUCTION

With the recent progress in immunosuppression research, small bowel transplantation (SBT) has entered the clinical arena [1]. However, as with other types of organ transplantation, donor shortage is a problem with SBT [2]. One option for solving this problem is to use segmental SBT from a single donor to multiple recipients. Although a human has >5 m of small bowel, only 1 m of transplanted small bowel is required for recipients with short bowel syndrome [3]. Therefore, knowledge of differences in susceptibility to cold preservation injury and subsequent reperfusion injury between the proximal and distal small bowel is important for avoiding primary mal-function. Many factors regarding intestinal function and enzymes have been reported as useful parameters for determining intestinal viability [4-8]. However, there is no single factor for distinguishing transplant tolerability between the jejunum and the ileum. One reason for this is that despite the fact that the small bowel has barrier functions in addition to digestive and absorptive functions, little is known about changes in barrier function in small bowel grafts as a result of either cold preservation or reperfusion.

In this study, to understand the differences in mucosal barrier function between the jejunum and the ileum during early reoxygenation following cold preservation, the epithelial cell functions at rest and upon glucose stimulation and the permeability to

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Abbreviations: ANOVA, analysis of variance; KHB, Krebs Henseleit buffer; PEG, polyethylene glycol; rPD, potential difference at rest; PDgs, potential difference upon glucose stimulation; PSP, phenolsulphonphthalein; UW, University of Wisconsin.
non-absorbable markers were evaluated during reoxygenation. The segmental difference in time-related histologic damage during cold preservation was also evaluated.

MATERIALS AND METHODS

Animals

Thirty male Lewis rats (8-15 weeks of age) were purchased from Charles River (Kanagawa), housed in the animal care facility of Kurume University with controlled light/dark cycles, and allowed free access to food and water until the experiments were initiated. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research, the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH publication No. 86-23, revised 1985), and the guidelines of the Kurume University Institutional Animal Care and Use Committee.

Organ procurement and preservation

Rats were fasted for 12 hrs prior to surgery. Under ether inhalation anesthesia, small bowel grafts were procured by the modified method of Monchik et al. [9]. Briefly, a section of small bowel from 0.5 cm distal to the ligament of Treitz to 1 cm proximal to the cecum was isolated. After insertion of a 22-gauge vasoluminal catheter (AngiocathTM, Becton Dickinson, Sandy, UT) into the abdominal aorta, the graft was perfused in situ by a syringe pump (Microfeeder JP-SE WH., Furue Science, Tokyo,) with 20 ml University of Wisconsin (UW) solution (4°C). The small bowel and accompanying portal vein and superior mesenteric artery were then removed en bloc. The bowel lumen was manually flushed from the proximal end with 40 ml cold UW solution and the bowel immersed in cold UW solution. The grafts were then preserved in cold UW solution until the experiments were performed. Samples from the middle of the jejunum and the middle of the ileum were taken from each graft at 0 h after procurement (as a control), and at 12 and 24 hrs after procurement, and used for the experiments.

Potential differences at rest and upon glucose stimulation

Seven grafts were used for this experiment. Electrophysiologic measurements were performed as previously reported by Takeyoshi and coworkers [10]. Two specimens that contained no Peyer's patches, one from the ileum graft and the other from the jejunum graft, were mounted simultaneously onto an Ussing chamber (World Precision Instrument, Sarasota, FL). Each surface of the specimens was continuously perfused with 20 ml recirculating oxygenated (95% O2, 5% CO2) Krebs Henseleit buffer solution (KHB) consisting of 120 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 2.5 mM CaCl2, 25 mM NaHCO3, and 1.2 mM KH2PO4 at a pH of 7.4. The recirculating reservoirs were maintained at 37°C. Agar bridges separately connected the mucosal and submucosal compartments to voltage-sensitive and current-passing electrodes.

To evaluate the potential difference of the grafts during reoxygenation following cold preservation, the graft surfaces were perfused with KHB solution for 20 min. The potential difference at rest (rPD) was then recorded, and the change in the potential difference produced by active glucose uptake from the mucosal to the serosal side, after the addition of 10 mmol/L glucose to the mucosal side, was recorded continuously for 60 min.

The potential difference upon glucose stimulation (PDgs) was determined with the following formula: Potential difference upon glucose stimulation (mV/cm²)=(Maximum potential difference after adding glucose−rPD)/(surface area) where rPD was the resting potential difference (mV), the maximum potential difference after adding glucose (mV) was the highest value during the 60 min after glucose addition, and the surface area (cm²) was calculated with a pin circle diameter at the mounting site.

Permeability studies

Fourteen grafts were used for this experiment. Two specimens, one from the jejunum graft and the other from the ileum graft, were mounted simultaneously onto an Ussing chamber and were continuously perfused. Phenolsulfonphthalein (PSP), a small non-absorbable marker, or radioisotope-labeled polyethylene glycol (PEG) were used to measure the permeation rate from the mucosal side to the serosal side.

PSP permeation

After a 20-min reoxygenation period, 6 mg PSP were added to the mucosal side chamber and a 0.1-ml aliquot from the mucosal side chamber was immediately removed to determine the initial concentration of PSP on the mucosal side. Subsequently, 1-ml aliquots from the serosal side were removed 30
min after PSP addition on the mucosal side. The absorbance at 450 nm was measured using a U-2000 spectrophotometer (HITACHI, Tokyo), and PSP concentration values were determined according to the manufacturer’s manual.

The permeation rate of PSP from the mucosal to the serosal side was determined with the following formula: Permeation rate of PSP (%)=100 (PSPserosal) (Ve-serosal)/(PSPmucosal) (Ve-mucosal) where PSPserosal (mg/ml) is the concentration of PSP on the serosal side, PSPmucosal (mg/ml) is the initial concentration of PSP on the mucosal side, Ve-serosal (ml) is the recirculating volume in the serosal side chamber, and Ve-mucosal (ml) is the recirculating volume in the mucosal side chamber.

PEG permeation
After a 20-min reoxygenation period, 1 ml radiolabeled PEG solution was added to the mucosal side chamber. The solution contained 14C-polyethylene glycol (37 kBq/ml), 10 mg unlabeled PEG, and KHB solution to a total volume of 1 ml. After the addition of the radiolabeled PEG solution, a 0.1-ml aliquot from the mucosal side was removed to determine the initial radioactivity at the mucosal side. Subsequently, 0.1-ml aliquots from the serosal side were removed 30 min after addition of radiolabeled PEG solution. Each aliquot was placed into a vial containing liquid scintillation solution (ACS2, Amersham, Tokyo), and radioactivity was measured with a liquid scintillation counter (LSC1000, Aloka, Tokyo).

The permeation rate of PEG from the mucosal side to the serosal side was determined with the following formula: Permeation rate of PEG (%)=100 (PEGserosal) (Ve-serosal)/(PEGmucosal) (Ve-mucosal) where PSPserosal (dpm/ml)=the radioactivity of PEG on the serosal side, PEGmucosal (dpm/ml)=the initial radioactivity of PEG on the mucosal side, Ve-serosal (ml)=the recirculating volume in the serosal side chamber, and Ve-mucosal (ml)=the recirculating volume in the mucosal side chamber.

Histologic examination
Nine grafts were used. Samples were fixed in 10% formalin and stained with hematoxylin and eosin. The grade of tissue injury was determined by light microscopy (magnification X100) according to the criteria described by Park and coworkers [11]- grade 0, normal mucosa; grade 1, presence of subepithelial space on the villous side; grade 2, expansion of subepithelial space on the villous side; grade 3, epithelial lifting along the villous side; grade 4, denuded villi; grade 5, loss of the villous side; grade 6, either destruction of both the villous side and the crypt layer or an obvious finding of crypt layer infarction; grade 7, presence of transmucosal infarction; grade 8, transmural destruction of the tissue by infarction.

Statistics
Data from the Ussing chamber study were expressed as mean±SEM. Statistical analysis was based on one-way analysis of variance (ANOVA) or Student’s t test and considered significant at p<0.05. Histologic grade was analyzed statistically by the Mann-Whitney U test and considered significant at p<0.05.

RESULTS
The time-related rPD during cold preservation
The time-related rPD in the jejunum and in the ileum during cold preservation are shown in Table 1. There was no significant difference in rPD between the jejunum and the ileum at 0 hr and after 12 hrs of preservation. The rPD of the jejunum was signifi-

| TABLE 1. |
| Time-related potential difference at rest during cold preservation |

<table>
<thead>
<tr>
<th></th>
<th>rPD-0 h (mV/cm²)</th>
<th>rPD-12 h (mV/cm²)</th>
<th>rPD-24 h (mV/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jejunum (n=7)</td>
<td>3.5±0.8</td>
<td>3.1±0.2</td>
<td>1.4±0.3</td>
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<tr>
<td>Ileum (n=7)</td>
<td>4.1±0.8</td>
<td>3.3±0.2</td>
<td>1.9±0.3*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM.
rPD-0 h, 12 h, and 24 h: Transepithelial potential difference at rest, at 0 hr and after 12 hrs or 24 hrs of preservation.
*p<0.05 versus jejunum after 24 hrs of preservation.
cantly lower than that of the ileum after 24 hrs of preservation.

The time-related PDgs during cold preservation

The time-related PDgs in the jejunum and in the ileum during cold preservation are shown in Table 2. There was no significant difference in PDgs between the jejunum and the ileum at 0 hr or after 12 hrs of preservation. The PDgs of the jejunum was significantly lower than that of the ileum after 24 hrs of preservation.

The time-related permeation rate of PSP during cold preservation

The time-related 30-min permeation rates of PSP in the jejunum and in the ileum during cold preservation are shown in Table 3. There was no significant difference between the permeation rate of PSP in the jejunum and the ileum at 0 hr preservation. However, at both 12 hrs and 24

<table>
<thead>
<tr>
<th>TABLE 2.</th>
<th>Time-related potential difference upon glucose stimulation during cold preservation</th>
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<td>PDgs-0 h (mV/cm²)</td>
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<tr>
<td>Jejunum (n=7)</td>
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</tr>
<tr>
<td>Ileum (n=7)</td>
<td>3.5±0.3</td>
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</table>

Data are expressed as mean±SEM.
PDgs-0 h, 12 h, and 24 h: Transepithelial potential difference at glucose stimulation 0 hr and after 12 hrs or 24 hrs of preservation.
*p<0.05 versus jejunum after 24 hrs of preservation

<table>
<thead>
<tr>
<th>TABLE 3.</th>
<th>Time-related permeation rate of phenolsulfonphtalein during cold preservation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>PSP-0 h (%)</td>
</tr>
<tr>
<td>Jejunum (n=7)</td>
<td>5.9±0.6</td>
</tr>
<tr>
<td>Ileum (n=7)</td>
<td>5.3±0.5</td>
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</tbody>
</table>

Data are expressed as mean±SEM.
PSP-0 h, 12 h, and 24 h: Thirty-min permeation rate of phenolsulfonphthalein at 0 hr and after 12 hrs or 24 hrs of preservation.
*p<0.05 versus jejunum after 12 hrs of preservation
†p<0.05 versus jejunum after 24 hrs of preservation

<table>
<thead>
<tr>
<th>TABLE 4.</th>
<th>Time-related permeation rate of polyethylene glycol during cold preservation</th>
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<tbody>
<tr>
<td></td>
<td>PEG-0 h (%)</td>
</tr>
<tr>
<td>Jejunum (n=7)</td>
<td>7.5±2.1</td>
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<tr>
<td>Ileum (n=7)</td>
<td>5.9±2.0</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM.
PEG-0 h, 12 h, and 24 h: Thirty-min permeation rate of polyethylene glycol at 0 hr and after 12 hrs or 24 hrs of preservation.
*p<0.05 versus jejunum after 24 hrs of preservation
SEGMENTAL DIFFERENCE IN COLD STORAGE SMALL BOWEL

TABLE 5.
Time-related tissue damage grading during cold preservation

<table>
<thead>
<tr>
<th>Histologic damage</th>
<th>Grade 0</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Grade 4</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>Jejunum-0 h (number)</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>Ileum-0 h (number)</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>Jejunum-12 h (number)</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>7</td>
<td>0</td>
<td>2.77±0.44</td>
</tr>
<tr>
<td>Ileum-12 h (number)</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>5</td>
<td>0</td>
<td>2.56±0.52</td>
</tr>
<tr>
<td>Jejunum-24 h (number)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>0</td>
<td>4.00±0.00</td>
</tr>
<tr>
<td>Ileum-24 h (number)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>8</td>
<td>3.89±0.33</td>
</tr>
</tbody>
</table>

Histologic damage is graded by Park's criteria[16].
Values are expressed as mean±SEM.
Jejunum-0 h, 12 h, 24 h: Jejunum at 0 hr and after 12 hrs or 24 hrs of preservation
Ileum-0 h, 12 h, 24 h: Ileum at 0 hr and after 12 hrs or 24 hrs of preservation

hrs of preservation, the permeation rate of PSP in the jejunum was significantly higher than that in the ileum.

The time-related permeation rate of PEG during cold preservation

The time-related 30-min permeation rates of PEG in the jejunum and in the ileum during cold preservation are shown in Table 4. There was no significant difference between the permeation rate of PEG in the jejunum and the ileum at 0 hr or after 12 hrs of preservation. However, after 24 hrs of preservation, the permeation rate of PEG in the jejunum was significantly higher than that in the ileum.

The time-related histologic damage

The time-related histologic damages in the jejunum and in the ileum during cold preservation are shown in Table 5. There was no significant difference in time-related histologic damage between the jejunum and the ileum at 0 hr, 12 hrs, or 24 hrs of preservation.

DISCUSSION

For successive segmental SBT from a single donor to multiple recipients, correct evaluation of segmental differences between the jejunum and the ileum is indispensable. Since the condition of the small intestine changes drastically from cold preservation to reperfusion, this evaluation should be performed during reoxygenation at the earliest. An intestine that is exposed to preservation and subsequent reperfusion injury at revascularization is most likely damaged, even when the warm and cold ischemic times are short. The characteristic histologic finding during reoxygenation is denuding or destruction of the villi [12,13]. Villous regeneration takes at least 2 or 3 days [10].

The mucosal barrier system in the small bowel has three main components: the mucus layer, in which mucin is bound by IgA; the epithelial cell layer; and the interepithelial space, which contains the junction complexes and intraepithelial lymphocytes [14]. The crypt patches and Peyer’s patches are also important sites that contain intraepithelial T cell precursors and IgA precursors. In this system, epithelial cells are not only structural components, but also play very important roles in secreting mucin and communicating with adjacent epithelial cells to maintain the junction complexes that induce intraepithelial lymphocytes. Therefore, the morphologic changes that are observed in the small intestine villi during reperfusion may seriously impair mucosal barrier function. When this occurs, unbound antigens adhere easily to the mucosa or may penetrate through the barrier system into the systemic circulation. Such pathogens not only injure the small intestine itself but also play a critical role in the development of serious conditions such as systemic inflammatory response syndrome and bacteremia, which is sometimes fatal [15]. Therefore, the mucosal barrier function of the intestine, rather than the digestive and absorptive functions alone, should be evaluated at an early phase after revascularization or reoxygenation.

In our previous study, the significantly higher permeation rate of PSP and the lower potential difference upon glucose stimulation in the jejunum compared to the ileum were recognized after a 10-min reoxygenation period following 24 hrs cold preservation, and we concluded that the ileum is more resistant than the jejunum to cold preservation.
injury [16]. However, the histological damage of both the jejunum and the ileum after 24 hrs cold preservation was almost grade 4, which is considered irreversible after transplantation [12]. Therefore, transplantation is necessary to evaluate the difference in tolerability between the jejunum and the ileum. Reperfusion injury begins with lipid peroxidation of the cellular membrane, which occurs within a few seconds after reoxygenation is initiated and continues for several hours [17]; therefore, in the present study, the potential difference and permeation studies were evaluated at 20 min after reoxygenation instead of 10 min following cold preservation.

We evaluated the segmental difference in the rPD. In both the jejunum and the ileum, the decrease of rPD was time-dependent. The rPD is produced by the difference of electrical charge between the mucosal (luminal) side and the serosal side; therefore, the time-related decrease of rPD is considered to be the result of intracellular Na+ accumulation, which was manifested as epithelial edema in the histologic findings described previously [12]. However, physiologically, the rPD of the jejunum is lower than that of the ileum, and the segmental difference between the jejunum and the ileum does not imply impaired viability. We also evaluated the PDgs. The PDgs is regulated by both chemical and physical forces [18]. The chemical force is provided by the Na+ gradient across the brush border, which is independent of the intracellular Na+ concentration that is maintained at low levels through the action of the Na-K ATPase pump on the basal membrane of epithelial cells. The physical force is provided by the electrical potential of the brush border membrane. Although the brush border transport mechanism is much more efficient than the basal-lateral exit pump, these two forces are not dependent, but cooperative. Therefore, the PDgs measurement results indicate that time-related impairment of epithelial function occurs faster in the jejunum than in the ileum. However, the differences in rPD and PDgs between the jejunum and ileum occurred after 20 min of reoxygenation following 24 hrs of preservation when the histological grades of both the jejunum and ileum were almost grade 4, which is an irreversible state after transplantation.

In measurements using a non-absorbable substance, the permeation rate of PSP (molecular weight, 345) and of PEG 4000 (molecular weight, 4000) in the jejunum was higher than in the ileum after 12 hrs of preservation. The histological grade of the jejunum and ileum after 12 hrs of preservation was either grade 2 or grade 3, which are both considered to be reversible after transplantation [12]. The time-related segmental difference in permeation rate between PSP and PEG suggests that as the particle size decreases, the permeation rate accelerates. Since physiologically the jejunum, which has aqueous channels approximately 7.5 to 8 angstroms in diameter, is more leaky than the ileum, which has channels 3 to 3.5 angstroms in diameter [19], the possibility that the jejunum is impaired earlier than the ileum cannot be concluded from our results. However, from the viewpoint of pathogen penetration and translocation, the mucosal barrier of the jejunum is impaired earlier than that of the ileum.

A recent report indicated that the basal-lateral membrane has an important function in the maintenance of junction complexes [20], which are the essential factors in mucosal barrier function. The observation that the accentuation of small particle permeation occurred earlier than the reduction of potential difference implies that the impairment of basal-lateral membrane function prior to that of brush-border membrane function in the jejunum might occur earlier than in the ileum.

Moreover, in this study, the grafts were evaluated under newly-reoxygenated conditions without chemical or gaseous mediators. In the clinic, grafts are exposed not only to reoxygenation stress but also to subsequent endothelial cell injury, which influences excretion of NO, endothelin secretion, activation of the arachidonic acid cascade, cytokine production, and expression of adhesion molecules [21,22]. This series of events evoke vasoconstriction, platelet aggregation, and/or neutrophil chemotaxis and activation. Such a graft in the clinical situation is more damaged than the grafts in this experiment; therefore, larger substances that evoke serious systemic injury might penetrate or translocate to the jejunal mucosal layer more readily than to the ileal layer.

CONCLUSION

Based on histologic grade, there was no significant difference between the jejunum and the ileum. However, our functional studies demonstrated that, in the rat small bowel, the ileum is more resistant than the jejunum to cold preservation. Therefore, when preservation time is prolonged, only the ileum should be transplanted.

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