NUMBER OF SEROTONIN POSITIVE CELLS AND ACUTE CELLULAR REJECTION IN THE EARLY PERIOD AFTER SMALL BOWEL TRANSPLANTATION IN PIGS

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Background. Small bowel transplantations (SBT) are being increasingly performed to treat patients with irreversible intestinal failure or short-bowel syndrome. Histologic evaluation of small bowel allograft biopsies is important for the diagnosis of acute cellular rejection (ACR). Serotonin (5-hydroxytryptamin) is a biogenous amine of which up to 95% is present in the enterochromaffin cells of the gastrointestinal tract. The aim of our study was to analyze rejection and number of serotonin positive cells in the intestinal graft biopsy samples early after SBT in pigs.

Material and methods. 24 pigs were used and divided into 4 groups. Group A, autologous SBT (n = 3) as a control group; group B, allogeneic SBT with tacrolimus monotherapy (n = 7); group C, allogeneic SBT immunosuppressed with tacrolimus and sirolimus (n = 8); and group D, without immunosuppression (n = 6). Observation period was 30 days. Mucosal biopsies were obtained on days 0, 3, 5, 7, 10, 14, 20, 28 after transplantation. ACR was classified according to standardized grading schema on a scale of indeterminate, mild, moderate, and severe. Serotonin positive cells were quantified as the number of positive cells in 20 high power fields.

Results. There were no significant differences in the number of serotonin positive cells and different grades of ACR. In our experiment the number of serotonin positive cells was not a sensitive marker of ACR in the early period after small bowel transplantation.

INTRODUCTION

Intestinal transplantation is becoming increasingly utilized as therapy for irreversible intestinal failure associated with failure or severe complications of total parenteral nutrition. Short-term graft and patient survival after small bowel transplantation (SBT) have improved dramatically during the past decade, especially after the introduction of tacrolimus and rapamycin¹. Despite potent immunosuppressive protocols, acute cellular rejection (ACR) remains a frequent complication and also represents the major cause of intestinal graft failure after SBT. Currently, evaluation of small bowel biopsy samples represents the optimal method to identify ACR. Because intestinal rejection, if not treated promptly, can rapidly increase in severity, is regularly followed by sepsis, and can result in graft loss or death, early detection and treatment are essential. In a clinical setting, ACR and infectious complications with following sepsis are mutually connected. The cause is due to the loss of the mucosal barrier during rejection which makes the bowel susceptible to bacterial translocation with endotoxemia². The rejection changes are often patchy, and thus multiple biopsy samples are required. Early after transplantation the ileostomy makes it possible to obtain the repeated endoscopic biopsies. Within the first year after SBT, ileostomy is closed in cases with good graft function, and the regular monitoring of the graft becomes more complicated. Recently, serotonin

(5-hydroxytryptamin) has been suggested as a possible marker of small bowel injury³. The use of serotonin for predicting the damage of intestinal mucosa after SBT is based on the fact that serotonin is present mainly in the enterochromaffine cells of the gastrointestinal tract mucosa. The objective of our study was to analyze rejection and the number of serotonin positive cells in intestinal graft biopsy samples early after SBT in pigs.

MATERIAL AND METHODS

Animals

Inbred line of pigs, female, weighing 35–40 kg were used according to the Czech Law and the standards for handling laboratory animals as stipulated by Act 246/92 Coll⁴. All the animals were kept in quarantine for 14 days. After that 24 pigs were randomly divided into 4 groups. Group A, autologous SBT (n = 3) as a control group; Group B, allogeneic SBT with tacrolimus monotherapy (n = 7); Group C, allogeneic SBT immunosuppressed with tacrolimus and sirolimus (n = 8); and Group D, without immunosuppression (n = 6).

Anesthesia and operation

Prior to graft harvesting and/or transplantation, the animals were pre-treated (stresnil 7 mg/kg, ketamine 10mg/kg, atropine 0.001 mg/kg), with subsequent an-

Graph 1. Survival time in groups of the animals.
Legend: POD, postoperative day.

Fig. 1. Mild and moderate acute rejection is characterized by inflammatory infiltrate in the lamina propria and by evidence of crypt damage with increasing number of apoptosis (H&E, original magnification x400).

Fig. 2. Severe acute rejection is distinguished by a marked degree of crypt injury with mucosal destruction (H&E, original magnification x200).

esthesia induction (hypnomidate 1 mg/kg + fentanyl 5 ml). Throughout the operation, anesthesia was maintained with a mixture of anesthetics (isofluran 1–2% + fentanyl 0.2 ml/kg) used on a standard basis in our laboratory, with ventilation controlled by an SV Siemens 900 system. Together with pretreatment, all the animals were given veterinary amoxicillin-clavulanate at a dose of 0.05 mg/kg. Another two doses were administered at 24 hour intervals after the first dose. Pain in the postoperative course was controlled by analgesia (butorphanol 0.2 mg/kg). Technical aspects of the operation procedure have been described in more detail earlier⁵.

Postoperative monitoring and immunosuppression

The observation period was 30 days. Postoperative care included daily monitoring of general condition (appearance of the stoma, weight, temperature, output and nature of stool). All the animals were given 2–l lactate Ringer's and 500 ml 20% glucose solution daily during the 3 postoperative days. Then they were given liquid food, and from the 4th or the 5th day they resumed a normal diet. No induction therapy or steroids were used. Monotherapy with tacrolimus was administered by gastric sonde in Group B to maintain a serum level of 15 ± 5 ng/ml. Double immunosuppressive therapy with tacrolimus and sirolimus was administered by the same way in Group C to maintain a serum level 7–10 ng/ml for tacrolimus, and 5–10 ng/ml for sirolimus.

Graph 2. ACR-score in different groups of animals within observation period.
Legend: ACR, acute cellular rejection; POD, postoperative day.

Graph 3. The number of serotonin positive cells in the different groups of animals.
Legend: POD, postoperative day.

Histopathological examination

Mucosal biopsies were obtained on days 0, 3, 5, 7, 10, 14, 20, and 28 using biopsy forceps. The biopsy specimens were fixed in 10% formalin, embedded in paraffin, cut at 3–4µm and stained with hematoxylin-eosin (H&E) and PAS. ACR was classified according to standardized grading schema^{6,7} on a scale of indeterminate (grade 0), mild (grade 1), moderate (grade 2), and severe (grade 3). The histologic criteria for grading of acute cellular rejection

(ACR) included a combination of infiltration by a mixture of mononuclear inflammatory cells, the extent of crypt injury, the increase in the number of crypt apoptotic bodies and distortion of the villous and crypt architecture.

Immunohistochemistry. Immunohistochemistry was performed on 4 m-thick paraffin sections of biopsy samples. The slides were deparaffinized in xylene and rehydrated in graded ethanol. After deparaffinization

Fig. 3. A high number of serotonin positive cells (redish-brown) in the mucosal biopsy sample with mild rejection changes (original magnification x200).

Fig. 4. Severe acute rejection with destruction of the intestinal mucosa and a low number of serotonin positive cells (original magnification x200).

and rehydration, the slides were cooked in a microwave oven using 0.01M citrate buffer pH 6.0 for target retrieval. Endogenous peroxidase was blocked by 0.3% H_2O_2 in 70% methanol for 30 minutes. The tissues were then preincubated with a 10% horse serum (Vector laboratories, Burlingame, CA) for 20 min to prevent nonspecific binding and FcR binding. Primary antibody (anti Serotonin, DakoCytomation, Denmark), diluted 50x, was applied for 30 minutes. Detection of monoclonal antibody was performed using biotinylated horse anti mouse IgG (Vector laboratories, Burlingame, CA) diluted 200x for 30 min. The specimens were then incubated with R.T.U. Vectastain Elite ABC Reagent for 30 min. Finally, specimens were stained with 3,3 diaminobenzidine (Serva, Germany) for 5 min and were counterstained with Harris's hematoxylin before they were embedded in Entellan (both from Merck, Germany).

Two main parameters were evaluated: ACR, and the average number of serotonin positive cells in 20 high power fields.

Statistics

The data are presented as means and SEM. Differences among groups were analyzed by Kruskal-Wallis one-way ANOVA followed by the multiple comparison method. All tests were two-tailed and $p < 0.05$ is considered statistically significant.

RESULTS

Survival

The best survival rate was attained in Group A. All animals in this group survived the observation period successfully and in 30 days were sacrificed. In Group D all animals died within 9 days. Statistically significant differences were demonstrated among Groups A, B, and C in contrast to Group D ($p < 0.01$; 0.05; resp. 0.01).

The data of survival time are summarized in Graph 1.

Graph 4. The number of serotonin positive cells according to the grade of the rejection episodes.

Histology

Altogether 153 biopsy samples of the small bowel mucosa were evaluated. In Group A, there were mild non-specific inflammatory infiltrates in the mucosa, but no increase in crypt epithelial apoptosis, and morphological features of ACR were absent. There was a lower number with milder score of episodes of ACR in group C than in Groups B and D (Figs. 1. and 2.). Statistically significant differences appeared on days 3, 5, and 7 postoperative ($p < 0.05$). All animals in Group D suffered from rejection from day 3. The episodes of ACR in this group rapidly progressed to the severe grade which was followed by sepsis and death (Graph 2.)

The average number of serotonin positive cells in different groups of animals during the observation period is summarized in Graph 3. There was a statistically significant decrease in Group D in comparison with Group C on days 3, 5, and 7.

We also evaluated the number of serotonin positive cells in connection with grade of ACR (Graph 4.). These

results were not statistically significant ($p = 0.437$). The differences in number of immunohistochemically serotonin positive cells are shown in Figs. 3. and 4.

DISCUSSION

Small bowel transplantations are being increasingly performed to treat patients with irreversible intestinal failure or short-bowel syndrome. Acute cellular rejection is the major cause of intestinal graft failure after transplantation. At this time, histological evaluation of the small bowel mucosa is the optimal method for identifying ACR. If not treated early, intestinal ACR can rapidly increase in severity and cause graft failure or death. Moreover, even mild intestinal dysfunction is associated with erratic absorption of immunosuppressive drugs, which can quickly destabilize even a previously well-functioning graft. Ileostomy is used to control the small bowel graft condition, with the regular sampling of mucosal biopsy for several months after SBT. If the post-transplant development is favourable, the ileostomy is closed within 1 year from SBT, and the monitoring of graft function becomes much more difficult. Therefore, there has been an intense effort to discover some other markers to monitor the intestinal graft during the past decade. Serotonin has been recently reported as a possible marker of ischemic/reperfusion injury in animal model^{8,9}. A limited number of studies have discussed serotonin and ACR¹⁰.

In this experimental study we evaluated the changes in the number of serotonin positive cells in relation to acute cellular rejection in early phase after SBT. We predicted there would be differences in number of serotonin positive cells among the different grades of rejection. Surprisingly, we did not establish a statistically significant difference in the number of serotonin positive cells in the groups according to the various grades of rejection. We speculate that one of the reasons for this could be the size of the biopsy sample. Moreover, the majority of serotonin positive cells are located in the lower part of the mucosa and the precise orientation of a small biopsy sample is therefore crucial. In our study, the biopsy samples were first cut and stained in H&E and PAS for diagnosis of rejection changes, and the remaining tissues were used for serotonin detection. The main limitation of our study was the fact that a portion of the samples was small without optimal orientation of the mucosal tissue in paraffin blocks, and they showed only the remaining upper part of the mucosa. Another reason for our results could be the immunohistochemistry per se. This method probably is not sensitive enough to analyze the quantity of mate-

rial in the cells, and also shows the same positive staining above a certain level of serotonin granules regardless of the quantity present. Maybe use of the quantitative method would yield different results.

CONCLUSION

There were no significant differences in the number of serotonin positive cells and the different grades of ACR. The number of serotonin positive cells was not a sensitive marker of ACR in the early period after small bowel transplantation in pigs.

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