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Intestinal distribution of hyaluronan in small bowel allografting in the rat

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Abstract. Hyaluronan (hyaluronic acid; HA) was demonstrated and quantified in small bowel tissue at different times after small bowel transplantation. Semiallogeneic or semisyngeneic rat models were used to elicit either unidirectional graft rejection or graft-versus-host disease (GVHD). In normal rat small bowel, HA was present in the villous lamina propria and around medium-sized vessels in the interstitium of the crypt area. During graft rejection a cellular infiltrate and edema appeared in the lamina propria in the crypt area where an accumulation of HA was also demonstrated. There was progressive accumulation of HA in the small bowel during rejection, and on day 6 there was a threefold increase compared to the values in syngeneic grafts. The increase in tissue HA was paralleled by an increase in the total water content of the rejecting graft. In specimens from animals suffering from GVHD, no significant changes in water or HA content and distribution were observed until day 12. The data suggest that accumulation of HA might contribute to the pathophysiology of the transplantation edema and that HA might be of potential diagnostic value in differentiating between graft rejection and GVHD.

Key words: Small bowel transplantation, in rats – Rat, small bowel transplantation, hyaluronan – Hyaluronan, small bowel transplantation – Intestinal transplantation, in rats

Introduction

Hyaluronan (hyaluronic acid; HA) is a linear polysaccharide built up of repeated units of the disaccharide N-acetyl-glucosamine-glucuronic acid. The properties of HA are important in water homeostasis, as HA is a ubiquitous molecule in the interstitium, attracting water by osmotic forces and resisting water flow [3]. HA is observed in large quantities in the connective tissue of the lamina propria, where it may be assumed that it contributes to the stability of the interstitial environment during the immense flow gradients that occur as part of the resorptive function of the bowel [5].

A number of mediators likely to operate in inflammation have been reported to enhance HA synthesis in mesenchymal cells [4, 9, 11, 27]. In previous studies, an increase in the HA content of interstitial tissues in rejecting kidney and heart grafts has been described [7, 8]. In this study we have analyzed the accumulation and distribution of HA in grafts and in the recipient's own intestine after small bowel transplantation in rats, and we have correlated the findings to the interstitial edema induced by rejection in order to evaluate the early connective tissue response during small bowel graft rejection.

Materials and methods

Animals

Inbred male Lewis and Lewis x DA F1-hybrid rats (originally obtained from Möllegaard, Skensved, Denmark) were bred in our own animal quarters. The animals weighed 190–270 g at the time of transplantation. They had free access to a standard pellet diet and tap water.

Operative procedure

The graft consisted of the entire small bowel, isolated on a vascular pedicle that comprised the portal vein and mesenteric artery. After heparinization of the animal (300 IU i.v.), the graft was flushed at a fixed pressure of 40 cm H_2O with a histidine-buffered perfusion solution [12]. The left kidney of the recipient was removed and the graft was then anastomosed to the renal vessels using a nonsuture cuff technique previously described [25]. The oral end of the graft was closed blindly and the distal end adapted with an end-to-side anastomosis to the distal ileum. Anesthesia for transplantation and sacrifice was given via an intraperitoneal injection of chloral hydrate.

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Groups

Group 1. Semiallogeneically transplanted animals (using Lewis x DA F1-hybrids as donors and Lewis rats as recipients, thus leading to unidirectional rejection) were sacrificed on days 2 ($n = 5$), 4 ($n = 5$), and 6 ($n = 4$).
Group 2. Syngeneically transplanted Lewis rats (control group) were sacrificed on days 2 ($n = 4$), 4 ($n = 4$), 6 ($n = 3$) and 12 ($n = 3$).
Group 3. Semisyngeneically transplanted animals (with Lewis rats as donors and Lewis x DA F1-hybrids as recipients, thus leading to unidirectional GVHD) were sacrificed on days 6 ($n = 4$) and 12 ($n = 4$).

Tissue specimens

Specimens from grafts and from the recipient's own small bowel were taken from the distal jejunum. One section was placed in buffered 4% formaldehyde, pH 7.3, with 1% cetylpyridinium chloride to achieve precipitation of the tissue HA [20], and stored at room temperature until paraffin-embedded and processed for staining to localize HA in the tissue. Another part was analyzed for the total HA and water content, and a third part was placed in Histicon (Histolab, Gothenburg, Sweden) and later snap-frozen for peroxidase-antiperoxidase staining. Animals with clinical signs of technical complications such as vascular thrombosis, ileus, and infections were excluded at the time of exsanguination.

Calculation of water content and extraction of tissue HA

The specimens were weighed immediately on filter paper at room temperature – wet weight (w.w.) – and again later after lyophilization at – 80 °C for 4 days – dry weight (d.w.). The relative water content was calculated according to the formula $100 \times (1 - \text{d.w./w.w})$. The extraction and analysis of HA was performed according to techniques described previously [8, 24]. In brief, HA was first extracted from the lyophilized dried tissue with 0.5 M NaCl. Approximately 20 mg of the material was extracted with 2 ml of the buffer by constant shaking at 4 °C for 16 h. The supernatants were recovered and the HA concentrations analyzed in duplicate using a radiometric assay (Pharmacia Diagnostics, Uppsala, Sweden) according to principles previously outlined [24].

Staining of HA

An avidin-enzyme, biotin-protein system was used for the detection of HA in the tissue. The method is based on the specific interaction between HA and the protein core of the cartilage proteoglycan [10]. Biotin-labelled protein was incubated on dehydrated, 5-µm-thick, paraffin-embedded sections. After the addition of an avidin-peroxidase complex to the sections, the bound enzyme, i.e., the HA of the tissue, was demonstrated after developing with ethyl carbazole [20].

Immunohistochemical staining

The immunohistochemical staining was performed according to the method described by Sternberger [23]. Mouse monoclonal antibody clones OX6 (reactive with the constant region of rat class II antigen) [17] and W3/13 (reactive to a glycoprotein present on all T cells and also in minor amounts on some other cells, including granulocytes [2] were used. Goat anti-mouse IgG (ATAB, Scarborough, Me., USA) was used as a secondary antibody (diluted 1:40). Each specimen was also processed for hematoxylin and eosin staining.

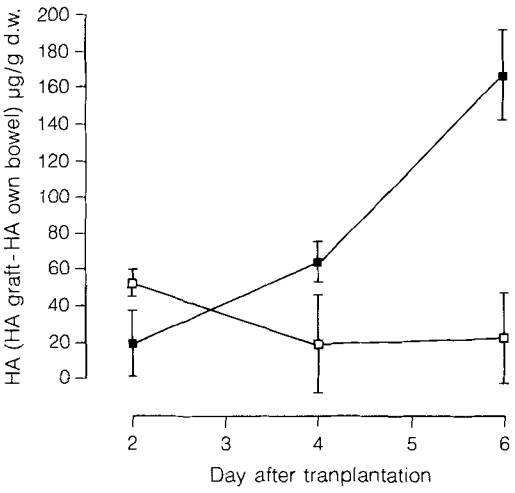

Fig. 1. HA content in the graft minus HA content in the recipient's own bowel (Δ HA) in semiallogeneically (rejection; ■) and in syngeneically (control; □) transplanted animals on days 2, 4, and 6 after transplantation

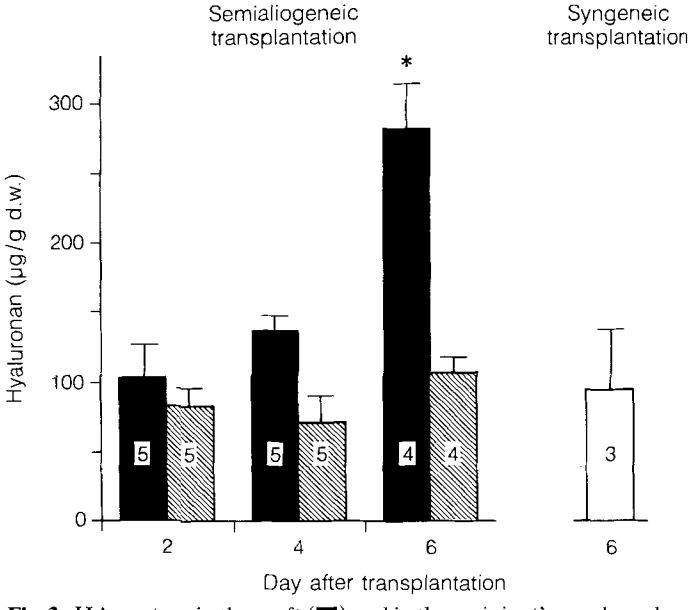

Fig. 2. HA content in the graft (■) and in the recipient's own bowel (▨) in semiallogeneically transplanted control animals during graft rejection, as well as that in grafts of syngeneically transplanted animals on day 6 after transplantation (□). Figures in the bars indicate the number of animals. * $P < 0.005$

Statistical analysis

All data are given as mean ± standard errors of the mean (SEM). Student's paired and unpaired t-test was used for statistical analysis. A difference at the 5% level was considered significant.

Results

Clinical signs of rejection were noted as a palpable mass of distended small bowel by day 6 in animals receiving semiallogeneic grafts. Clinical signs of GVHD were seen as a slight erythema on day 6 in animals receiving semisynge-

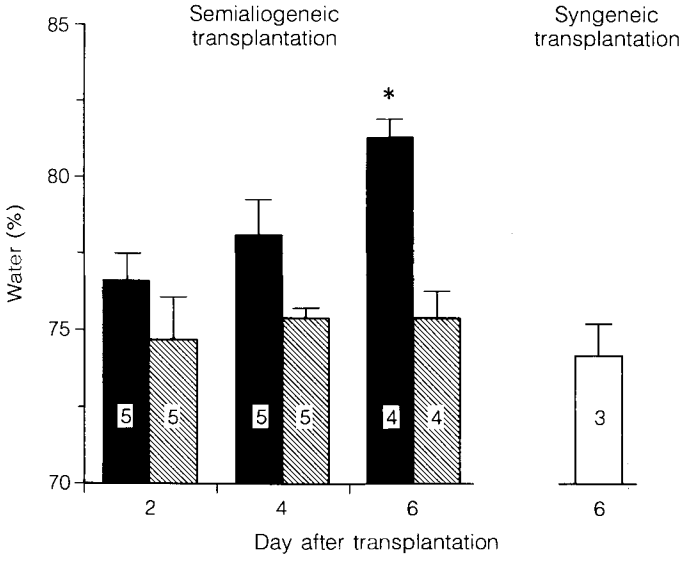

Fig. 3. Water content in the graft (■) and in the recipient's own bowel (▨) in semiallogeneically transplanted animals during graft rejection and in the grafts of syngeneically transplanted control animals (□) on day 6 after transplantation. Figures in the bars indicate the number of animals. * $P < 0.005$

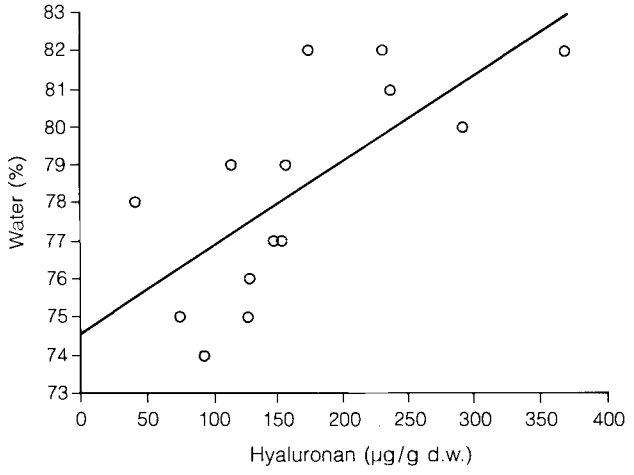

Fig. 4. Correlation between water content and amount of HA in the grafts of animals undergoing rejection on days 2, 4 and 6

neic grafts; by day 12 the skin reaction was more evident and associated with weight loss and general signs of malaise. The animals with syngeneic grafts remained well over the observation period.

HA content in the small bowel

Generally, there was a higher content of HA in the graft than in the recipient's own small bowel (Fig. 1). A progressive increase in the tissue content of HA was observed in rejecting grafts after semiallogeneic transplantation during the observation period (Figs. 1, 2).

On days 4 and 6 there were 90% and 300% increases, respectively, in tissue HA content in the graft and in the recipient's own small bowel. No progressive increase in HA content in the graft was noted in animals after syngeneic transplantation (Fig. 1) or in animals with grafts that entailed GVHD compared to that in the recipient's own small bowel (Table 1).

Water content in the small bowel

In the rejection group there was an increase in the total water content of the graft by day 6 (Fig. 3). In the recipient's own bowel and in grafts of syngeneically transplanted control animals, no such increase was observed. The graft water content during rejection correlated well with the increase in tissue HA ($r = 0.72$, $p < 0.01$; Fig. 4). The water content in the graft and in the recipient's own bowel during GVHD did not differ significantly (Table 1).

Morphology

In the normal small intestine there was a sparse infiltrate of inflammatory cells in the lamina propria. There was no, or only very slight, MHC class II expression in crypt epithelial cells in the jejunum (data not shown). The lamina propria of the crypt area was thin and there was virtually no measurable distance between the basement membrane of the crypts and the inner part of the muscularis propria (Fig. 5 A). There was positive staining for HA in the lamina propria of the villi and around medium-sized vessels in the crypt area, but also in the thin interstitium of the subserosa.

The morphology of syngeneic transplants differed only slightly from that of the recipient's own bowel, with a minor, but discernible, increase in the width of the crypt muscle distance (Fig. 5 B).

In grafts undergoing rejection, an increasing number of infiltrating inflammatory cells – many of which were T cells (W3/13 positive) – were seen, predominantly in the widened lamina propria of the crypt area and in the subserosa. The distance between the basement membrane of the crypts and the muscularis propria was greatly enhanced (Fig. 5 C), indicative of interstitial edema. There was also intense MHC class II staining of the crypt epithelial cells. The staining for HA was considerably increased in the lamina propria of the crypt area and also along the widened subserosa, both regions with a heavy accumulation of inflammatory cells.

The morphology of animals undergoing GVHD has been described previously [17]. A moderate increase in crypt cell class II expression of the graft and the recipient's own bowel was seen, as well as cellular infiltrates along the serosa of the graft. There was no pronounced redistribu-

Table 1. Hyaluronan (HA) and water content in graft and in own small bowel in animals receiving semisyngeneic grafts leading to unidirectional GVHD 6 and 12 days after transplantation

Tissue	Days post-transplantation	*n*	HA (μg/g dry weight)	Water (%)
Graft	6	4	65 ± 13	76.8 ± 0.3
Own bowel	6	4	105 ± 19	77.4 ± 0.5
Graft	12	4	96 ± 19	77.4 ± 0.4
Own bowel	12	4	96.5 ± 5	78.1 ± 0.5

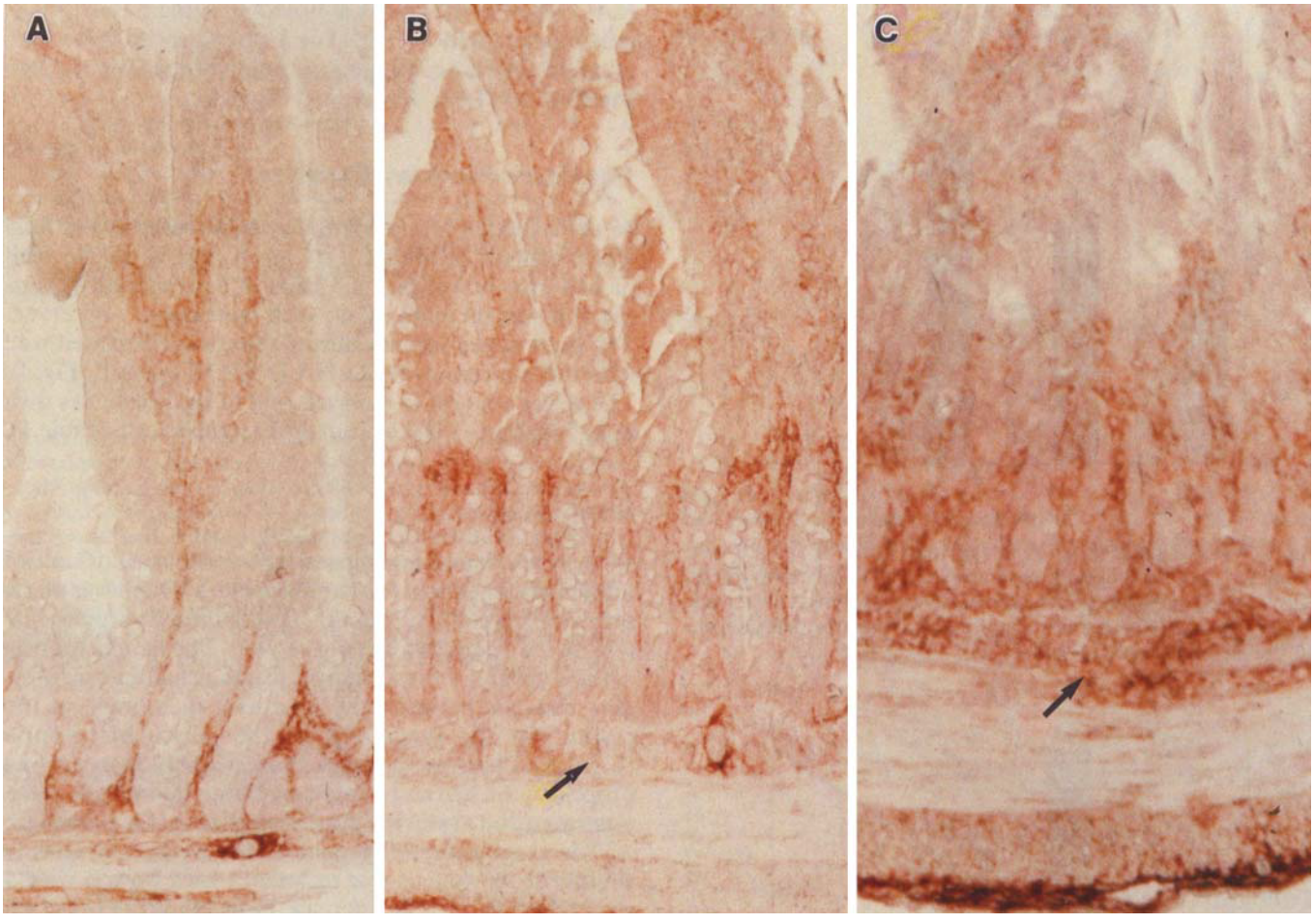

Fig. 5 A–C. HA staining, 6 days after transplantation. **A** In the recipient's own bowel; **B** in grafts of syngeneically transplanted control animals; and **C** in grafts of semiallogeneically transplanted animals during rejection. Note the widened crypt area and muscularis propria space *(arrows)* and the increased staining in this area and in the subserosa in the rejecting graft

tion of, or increase in, mononuclear cells in the lamina propria, and the HA content did not display any evident alterations compared with the normal small bowel (data not shown).

Discussion

In this study we have demonstrated the presence of HA in healthy small bowel tissue in rats and we have followed the accumulation of this glucosaminoglycan in small bowel tissue during rejection and GVHD. The distribution of HA in the normal rat bowel, as described here, corresponds in most respects to that previously reported in human specimens [5]. Accordingly, staining was observed in the lamina propria in the villi, where HA may play an important role in the normal physiology of the interstitium [5]. A progressive increase in graft tissue HA was found during graft rejection. The accumulated HA was mainly distributed in the space between the muscular layer and the crypt area and in the subserosa. No such reaction was observed during ongoing GVHD or in the control animals after syngeneic transplantation.

The threefold increase in HA content of the intestine by day 6 in grafts undergoing rejection paralleled an increase in the total tissue water. This link between HA accumulation and interstitial edema is analogous to observations of the rejection of heart and kidney grafts in the rat [7, 8]. Inflammatory interstitial edema has previously been attributed to an increased capillary leakage of water and solutes. Another essential, and probably coexisting, mechanism suggested by this and the previous studies is an increased ability of the interstitium to bind water by virtue of an increased content of HA. HA has thus been shown to have unique water-binding properties, and the tissue content of HA has been said to be a major regulator of the hydration of the interstitium [1, 16, 18, 19].

Although edema is macroscopically evident in other solid organs undergoing rejection, it is less evident to the naked eye in small bowel allografts. One reason for this difference may be that newly synthesized HA, which, as observed in this study, is mainly located subepithelially in the lamina propria of the small bowel, might be eliminated by leakage into the intestinal lumen together with water. In contrast, the elimination of accumulated HA in rejecting kidney and heart grafts is dependent upon lymphatic drainage or enzymatic degradation [7, 8]. Assuming that mucosal leakage of HA is enhanced, measurements of HA in the bowel discharge may be an alternative, noninvasive, diagnostic method for the detection of rejection.

In fact, in a recent clinical case of a small bowel transplant in a 13-month-old child with severe short bowel syndrome, an ongoing rejection, verified by biopsies, was associated with a several hundred percent increase in HA in the luminal fluid (Knutson et al., manuscript in preparation). In this study, no HA accumulation was observed during the clinically overt phases of GVHD, suggesting that the inflammatory response of the target organ is crucial for the observed increase in HA.

The precise cellular source of the intestinal HA during rejection has not been revealed in this study. It is conceivable that the mesenchymal cells present in the intestinal tissue may be activated to enhance HA production via immunomediators released by the cells invading the transplant. This is supported by the obvious colocalization of inflammatory cells and the increased HA. Several cytokines and growth factors released during inflammation influence fibroblast activity [15]. Certain inflammatory products with fibroblast-activating properties, e.g., interleukin 1, platelet-derived growth factor, and epidermal growth factor, have actually been shown to stimulate synthesis of HA in fibroblasts in vitro [4, 9, 11]. Thus, several factors related to immunological and/or inflammatory responses during graft rejection may stimulate HA synthesis in the bowel.

The well-known capacity of the small bowel to elicit both vigorous graft rejection and GVHD has been thoroughly documented [14] and, facing a clinical problem, there may be diagnostic uncertainty as to which process is at hand. Our previous studies of graft rejection and GVHD have mainly focused on morphological and immunohistochemical findings [26]. However, in the early stages of the process, the findings are discrete and nonspecific, and they also appear in a focal rather than in a homogeneous manner, which makes morphological diagnosis uncertain. Different aspects of bowel function [6, 21] and the measurement of various factors in the serum [13, 22] have also been evaluated for potential use in diagnosis. The results from this study suggest that the determination of HA may serve as a method for early detection of rejection and for differentiation between rejection and GVHD.

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