Signs of increased leakage over the jejunal mucosa during gliadin challenge of patients with coeliac disease

B Lavö, L Knutson, L Lööf, B Odlind, R Hallgren

Abstract

Intestinal secretion rates of albumin, hyaluronan, and beta-2-microglobulin (beta2-micro) were determined under basal conditions and after gliadin challenge of coeliac patients and healthy controls by the use of a jejunal perfusion technique. A new tube system was used where a jejunal segment is isolated between balloons and then perfused with a balanced salt solution. Under basal conditions the secretion rate of albumin was similar in the patients and controls while the secretion rate of the glycosaminoglycan hyaluronan, a high molecular weight connective tissue component, was increased more than two times in coeliac patients. Beta2-micro was secreted in on average three-fold rates in coelias compared with controls. All three substances were secreted at a higher rate in patients with active disease than in those with inactive disease defined by morphological damage in small bowel biopsies. The concentrations in jejunal perfusion fluids relative to serum levels in the coeliac patients were for albumin 0-0007, beta2-micro 0-10, and for hyaluronan 1-94. Challenge with a single dose of gliadin into the jejunal segment gave within 60 min a significant, about two-fold, increase of the secretion rates of all three measured substances. The appearance of hyaluronan could reflect a gliadin induced mucosal oedema with an enhanced leakage from the interstitial/lymph fluid, rich in this glycosaminoglycan. The observed parallel increases in the jejunal secretion of albumin and beta2-micro after gliadin challenge are best explained by a similar mechanism.

In coeliac disease the small bowel mucosa of genetically predisposed individuals is damaged after ingestion of wheat gluten and similar proteins in rye and barley. The pathophysiological events that lead to the characteristic 'flat' mucosa with damaged enterocytes and infiltration of inflammatory cells into the epithelium and lamina propria have not been fully elucidated. The proposed mechanisms for the mucosal damage are primarily immunological including both reactions elicited by antigen-antibody complexes and cell mediated damage. It has been reported that gliadin challenge may induce histological changes of the mucosa as early as within two hours after gliadin administration. Studies of the actue pathological events induced by gliadin challenge, however, have been hampered by the difficulties in obtaining serial samples from the mucosa.

Inflammatory affection of the mucosa might influence the leakage from the capillary bed/interstitial fluid to the bowel lumen. The aim of this study was to elucidate this possibility by continuous measurements of the intestinal appearance of substances from plasma and/or lymph fluid under basal conditions and after local gliadin challenge of a perfused jejunal segment. This was made possible by the use of a recently developed multichannel tube with two occluding balloons allowing measurement of the secretion in an isolated small bowel segment. In this study we report the jejunal appearance rates of low and high molecular weight substances; albumin, beta2-microglobulin (beta2-micro), and hyaluronan. The appearance of albumin in the intestinal perfusion fluid should merely reflect the protein leakage from the blood circulation and the intestinal interstitial fluid. Hyaluronan, a high molecular weight glycosaminoglycan, is an important connective tissue component synthesised mainly by mesenchymal cells and present in virtually every tissue of the body. It is drained from tissues by the lymph and appears in the lymph fluid in concentrations much higher than in plasma. Thus, the appearance of hyaluronan in intestinal fluids might reflect a local synthesis in the intestinal wall and a leakage from, primarily, the lymph or interstitial fluid. The jejunal appearance of the low molecular weight protein beta2-micro should also reflect a passive leakage but, in addition, it might as well reflect a local intestinal production, since this protein is part of the HLA class I receptors present on all nucleated cells. An increased production of beta2-micro can therefore be anticipated during an increased turnover of cells.

Methods

Patients

Ten patients with coeliac disease were studied by the jejunal perfusion technique. They were a selection of treated patients with a good clinical response to a gluten free diet and patients with a bad response because of dietary negligence. Two untreated patients were also studied; they later responded favourably to a gluten free diet and had normalised their small bowel mucosa. Seven of the patients were given a local challenge with gliadin into the jejunal segment. One patient was investigated twice, with and without gliadin challenge. Data concerning age, sex, disease duration, serum gliadin antibody level, dietary status, and histopathological findings are given in Table I. Healthy controls (n=14) were
also investigated with jejunal perfusion; three were challenged with gliadin under conditions identical to those for the coeliac patients. The mean age of the controls was 27 years (range 22–32).

**Table I** Clinical data on patients with coeliac disease investigated by regional jejunal perfusion under basal conditions and after gliadin challenge

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Year of diagnosis</th>
<th>Diet</th>
<th>AGA</th>
<th>Small bowel histology</th>
<th>Gliadin challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>VF</td>
<td>66</td>
<td>F</td>
<td>1981</td>
<td>GFD</td>
<td>59</td>
<td>PVA</td>
<td>-</td>
</tr>
<tr>
<td>DA</td>
<td>51</td>
<td>M</td>
<td>1987</td>
<td>N</td>
<td>350</td>
<td>PVA</td>
<td>-</td>
</tr>
<tr>
<td>CL</td>
<td>41</td>
<td>M</td>
<td>1977</td>
<td>GFD</td>
<td>17</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>RJ</td>
<td>71</td>
<td>M</td>
<td>1960</td>
<td>GFD</td>
<td>70</td>
<td>PVA</td>
<td>-</td>
</tr>
<tr>
<td>KL</td>
<td>43</td>
<td>M</td>
<td>1987</td>
<td>N</td>
<td>300</td>
<td>SVA</td>
<td>+</td>
</tr>
<tr>
<td>VS</td>
<td>60</td>
<td>F</td>
<td>1979</td>
<td>GFD</td>
<td>200</td>
<td>PVA</td>
<td>-</td>
</tr>
<tr>
<td>KJ</td>
<td>33</td>
<td>F</td>
<td>1986</td>
<td>GFD</td>
<td>15</td>
<td>PVA</td>
<td>+</td>
</tr>
<tr>
<td>VO</td>
<td>42</td>
<td>M</td>
<td>1980</td>
<td>GFD</td>
<td>70</td>
<td>PVA</td>
<td>+</td>
</tr>
<tr>
<td>OP</td>
<td>57</td>
<td>M</td>
<td>1985</td>
<td>GFD</td>
<td>60</td>
<td>PVA</td>
<td>+</td>
</tr>
<tr>
<td>ES</td>
<td>40</td>
<td>F</td>
<td>1985</td>
<td>GFD</td>
<td>7</td>
<td>N</td>
<td>+</td>
</tr>
<tr>
<td>CL</td>
<td>41</td>
<td>M</td>
<td>1977</td>
<td>GFD</td>
<td>17</td>
<td>N</td>
<td>-</td>
</tr>
</tbody>
</table>

Abbreviations: Diet; GFD = gluten free diet, N = normal diet. AGA = gliadin antibody titre (IgA; normal values of healthy <48 units). Histology of duodenal biopsy; SVA = subtotal villous atrophy, PVA = partial villous atrophy, N = normal villous architecture. Gliadin challenge = - no gliadin, + = gliadin.

Gliadin challenge
Gliadin (crude gliadin, Sigma Chemical Co, St Louis, Mo), 15 (4) mg (SEM) was dissolved in 0-5 ml 70% ethanol and then mixed into 30 ml of the perfusion fluid. After a 40–60 min basal perfusion, gliadin was administered as an infusion to the segment during 20 minutes. After gliadin administration the perfusion was continued for another 120 min. The gliadin concentration in the perfused segment was calculated to be 0-2 mg/ml. Concentrations in that range have been found to give a reaction in cell culture experiments.11

**Analytical measurements**
14C-PEG was determined by liquid scintillation counting (LKB Rackbeta II, Wallac Oy, Turku, Finland) and 1 ml aliquots in duplicate for 15 minutes.12 Phenol red was measured spectro-photometrically (Hitachi spectrophotometer model 1011, Hitachi Ltd, Tokyo, Japan) at 520 nm after alkalinisation.13

In order to further assure that contamination of the effluent from pancreatic secretion did not occur, the trypsin content of the effluent was measured, using N-p-tosyl-L-arginine methyl-ester (TAME) as substrate, and determined spectro-photometrically at 247 nm.14 The total proteolytic activity of the effluent was also assessed using casein as substrate.15 Before analysis the samples were thawed on ice and 2 mmol phenylmethylsulphonyl fluoride (PMSF; Sigma Chemical Co), a serine protease inhibitor, was added to avoid influences of...
minute amounts of proteases in the effluent.

The perfusion fluid samples were analysed in sequence and in duplicate for the content of albumin, hyaluronan and beta2-microglobulin. Hyaluronan was analysed according to the principles previously outlined. In this study, a modified technique was used as developed by Pharmacia Diagnostics (Uppsala, Sweden). Both of the tests are based on the use of specific hyaluronan binding proteins (HABP) isolated from bovine cartilage. In the modified test, the hyaluronan from the samples (100 μl) is allowed to bind "I-labelled hyaluronan binding proteins in solution for at least 60 min. The unbound "I-labelled hyaluronan binding proteins is then quantified by incubating with hyaluronan covalently coupled to Sepharose particles. After centrifugation and decanting, the radioactivity bound to the particles is measured. The two techniques give identical results. Albumin in the perfusion fluid (50 μl sample) and beta2-microglobulin (200 μl sample) were measured by double antibody radioimmunoassays (Pharmacia Diagnostics). Parallel standard curves were obtained for all substances by means of the respective standard curves mixed with either buffer or a constant volume of lavage fluid. The variability was less than 8% for all methods.

STATISTICAL ANALYSIS

Analyses of significance were performed by the use of Student's t-test on groups and paired values. The results are expressed both as jejunal fluid concentrations and appearance rates. The appearance rate was based on the steady infusion rate and calculated according to the formula:

\[ \text{concentration in perfusion fluid} \times 3 \text{ml/min} \times 60 \text{min}/10 \text{cm} = \text{amount/cm intestine/hour} \]

The study was approved by the Ethical Committee of the Medical Faculty, Uppsala University. Patients and controls gave their informed consent to participation in the study.

Results

The concentrations of beta2-microglobulin (beta2-micro), albumin, and hyaluronan in jejunal perfusion fluid from patients with coeliac disease and controls are given in Table II. Under basal conditions, the albumin concentrations were similar in patients and controls. Increased concentrations of hyaluronan were observed in the patient group but the difference did not reach statistical significance. The beta2-micro concentrations were on average trebled in the coeliacs compared with the controls (p<0.01). The basal appearance rates of the measured substances were calculated (Table II). The differences observed between the groups were not influenced by this calculation. Those patients who had active coeliac disease defined by histological findings on biopsy presented higher appearance rates of all substances, but the differences did not reach statistical significance (Table III).

The serum concentrations of albumin and hyaluronan in the coeliac group were 43 (0-7) (SEM) g/l and 44-5 (8-8) μg/1, respectively, and within the reference values at our laboratory; the normal range for albumin being 36–48 g/l and for HA 10–100 μg/l. The serum beta2-micro values tended to be slightly increased in the patient group, 1.8 (0.1) mg/l; the reference value at our laboratory is 1.1–2.1 mg/l. The ratios between the serum and jejunal fluid concentrations in the patients group were for beta2-micro 0.10, for albumin 0.0007 and for hyaluronan 1.94.

During challenge with gliadin the appearance rates of beta2-micro, albumin and hyaluronan started to increase at the same time and on average 40 minutes after administration of gliadin (Figs 2, 3). A tendency towards an earlier onset of the influx of beta2-micro was observed. The peak appearance rates of the measured variables were on average twice as high, compared with basal appearance rates, and reached a maximum 60 minutes after gliadin administration. The enhanced appearance rates of hyaluronan and albumin levelled off after that time but remained raised during the observation period of 120 minutes after the gliadin challenge. In contrast, the appearance rate of beta2-micro started to decrease after 60 min and returned to basal levels within the 120 min observation period (Fig 2). Gliadin challenge tests of healthy controls did not alter the appearance rates of the measured substances (data not shown). Among the coeliac patients the maximum appearance rates varied within narrow limits (Fig 4). The maximum increases, in absolute or relative terms, were apparently not related to differences in the histopathological activity of the disease.

Only two of the patients had minor subjective reactions during gliadin challenge. One reported a burning pain that he claimed to be the same as after accidental gluten ingestion. The other patient reported nausea. The other five patients reported no reactions. None of the controls complained of any reactions.

Discussion

The validity of the present investigation rests upon the quality of the perfusions. Previous investigations of small bowel secretion have mainly been performed by open perfusion systems or aspiration. The advantage of a

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**TABLE III** The jejunal secretion rates of beta2-micro, albumin and hyaluronan in patients with coeliac disease, subgrouped into patients with normal small bowel mucosa and patients with active disease as evidenced by villous abnormalities in small bowel biopsies

<table>
<thead>
<tr>
<th>Group</th>
<th>Beta2-micro</th>
<th>Albumin</th>
<th>Hyaluronan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μg/cm²/h</td>
<td>μg/cm²/h</td>
<td>ng/cm²/h</td>
</tr>
<tr>
<td>Non-active disease (n=3)</td>
<td>1.8 (0-3)</td>
<td>285 (89)</td>
<td>641 (59)</td>
</tr>
<tr>
<td>Active disease (n=8)</td>
<td>3.4 (0-9)</td>
<td>625 (99)</td>
<td>1709 (771)</td>
</tr>
</tbody>
</table>

Means (SEM).
Under basal conditions the measured albumin concentrations in the jejunal perfusion fluids of healthy controls and coeliac patients were about 0.1% of the serum concentrations, reflecting the degree of passive leakage of proteins from the plasma and interstitial fluid compartments to the jejunal fluid. The basal appearance rate of albumin in the whole group of coeliac patients was similar to that seen in controls. Patients with histologically active coeliac disease, however, presented an increased albumin appearance rate compared with coeliac patients with a normal histology of the mucosa. These findings support previous observations of intestinal albumin loss in patients with active coeliac disease.

The serum concentrations of hyaluronan vary between 10–100 μg/ml in healthy subjects, and patients with coeliac disease had similar serum values. The concentrations of hyaluronan measured in the jejunal perfusion fluid are close to hyaluronan concentrations in the blood circulation. Thus, a passive leakage from the plasma compartment cannot explain the high jejunal perfusion fluid concentrations of hyaluronan. A more likely source of hyaluronan is the lymph/interstitial fluid, as the concentration of hyaluronan is about 100 times higher in lymph fluid than in plasma. In contrast the albumin concentrations in lymph fluid are close to the serum concentrations of albumin. Provided that the appearance of hyaluronan and albumin in the jejunal fluid reflects a common mechanism, both substances should then be secreted into the intestinal lumen mainly from the lymph/interstitial fluid compartment and to a lesser extent from the plasma compartment. This would mean that the increase of the appearance rate of albumin and hyaluronan observed after gliadin challenge should reflect a lymph/interstitial fluid oedema with enhanced leakage of interstitial constituents. The main synthesis of hyaluronan is brought about by mesenchymal cells of the connective tissue. The demonstration in healthy individuals of a considerable hyaluronan appearance into the jejunal lumen might therefore reflect the normal rapid turnover of the intestinal mucosa. The increased basal appearance rate of hyaluronan observed in active coeliac disease may partly be the result of an enhanced local hyaluronan synthesis caused by stimulated cell replication of the mucosa. It seems less likely, however, that the increase of hyaluronan observed as early as 40 minutes after gliadin challenge should be caused by the induction of hyaluronan synthesis. Previously, Blanco et al have reported increased serum concentrations of beta2-micro in coeliac disease, a finding we were able to confirm in our patients. Furthermore, we observed a three-fold higher basal jejunal appearance rate of beta2-micro. Based on these findings in intestinal fluid, it is reasonable to
propose that the increased serum concentrations of beta2-micro in coeliac disease might at least partly reflect an enhanced production of this protein in the intestine. All nucleated cells are able to synthesise beta2-micro. An increased production of beta2-micro in the mucosa may reflect the increased enterocyte turnover which has been documented to be greatly enhanced in coeliac disease. An alternative explanation could be an increased production from invading cells, mainly lymphocytes. Beta2-microroglobulin is a low molecular weight substance (Mw 11800 daltons) and should appear more easily than albumin in the intestinal fluid. It was also calculated that the ratio between the concentration of beta2-micro in jejunal fluid in relation to serum levels was relatively higher in healthy controls than the corresponding ratio for albumin. The appearance rate of beta2-micro was on average doubled after glaidin challenge of coeliac patients. The increased influx of beta2-micro tended to precede the increased influxes of albumin and hyaluronan. Furthermore, in contrast with these substances, the enhanced appearance of beta2-micro was transient and at the end of the 120 min follow up period after glaidin administration, its appearance rate had returned to basal levels. These differences in the behaviour of the measured variables can possibly be explained if we assume that the leakage from the tissue is a transient phenomenon in our glaidin challenge model. In such a situation, differences in molecular sizes of the leaking substances might influence the kinetics of their transport to the jejunal lumen. Alternatively the rapid increase and decrease of beta2-micro could reflect the local tissue damage elicited by the glaidin challenge. Enterocyte damage has been reported to occur within two hours. In conclusion, we have shown that a very transient challenge of the small bowel with glaidin induces an increase in the appearance rates of beta2-micro, albumin, and hyaluronan in patients with coeliac disease but not in healthy controls. The appearance of these substances in increased amounts in the jejunal fluid points to a glaidin induced lymphoedema and an enhanced leakage from interstitial/lymph fluid. The perfusion technique used allows kinetic studies of the local intestinal reactions mediated by glaidin and it restricts the glaidin challenge in dose, time, and intestinal area.

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