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High Hepatocyte Growth Factor Levels in Faeces During Acute Infectious Gastroenteritis

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Hepatocyte growth factor (HGF) is a potent mitogen of mature epithelial cells which is produced after organ injuries and acts as a trigger for regeneration in the impaired organ. The aim of the present study was to investigate local production of HGF during infectious gastroenteritis. We measured the concentration of HGF in serum and faeces in 49 patients with acute infectious gastroenteritis (bacterium = 30, virus = 10, amoebae = 1, and probable infection = 8) at the time of referral to hospital and at convalescence ($n = 31$). The values were compared with normal healthy vaccination volunteers ($n = 11$) as well as patients with acute non-infectious diarrhoea ($n = 10$). The presence of HGF in faeces was confirmed by ELISA and Western immunoblot. HGF concentrations in faeces was significantly higher in the patients with infectious gastroenteritis compared to the control groups ($p < 0.0001$). Using a cut-off concentration of 20 ng/g, the overall sensitivity of faeces HGF to distinguish infectious gastroenteritis (bacterial, viral, probable infection) was 98% with a specificity of 100%. At convalescence all patients had normal values. There was no significant correlation between HGF concentrations in faeces and serum. Determination of faeces HGF may identify cases of transmittable diarrhoea requiring isolation at an early stage.

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INTRODUCTION

Diarrhoea is one of the most common manifestations of infectious diseases in the world. It is characterized by an increase in volume, fluidity or frequency of faecal discharges. The most common causes are viruses and, to a lesser extent, bacteria and parasites. In bacterial diarrhoea, mechanisms of disease include adherence of the organism to intestinal mucosal cells, with or without mucosal damage and invasion, and production of enterotoxins and cytotoxins (1). Clinical features vary greatly depending on the cause, and on the affected part of the intestine. Diarrhoea may also occur when the small and large intestine secrete, rather than absorb, electrolytes and water (2).

Hepatocyte growth factor (HGF) is a unique growth factor, which is unrelated to all other well-known polypeptide mitogens. It is a protein expressed in the mesenchymal cells such as lung macrophages and fibroblasts (3), Kupffer cells in the liver and leukocytes (4, 5). HGF is secreted in response to cell damage and appears to be important for the regeneration of certain organs and healing of wounds (6). It is a heterodimer, disulphide bonded heavy and light chains of approximately 60 and 30 kDa respectively, first synthesised as an inactive precursor (7). The precursor is cleaved to an active protein in the damaged organ by a specific activator (8, 9). HGF acts paracrinally, i.e. it affects adjacent cells, as well as endocrinally, i.e. it has a long-distance effect (10, 11). The target cells of HGF are fully developed epithelial cells (6). HGF is produced and is present in high concentrations at sites of organ damage (12). We have previously studied the systemic and local production of HGF in various infectious diseases and have observed high serum HGF concentrations during acute infectious diseases such as gastroenteritis (13). Simultaneous with enhanced systemic production of HGF, we found high HGF concentrations in cerebrospinal fluid during meningitis (14). Raised HGF concentrations in exhaled breath condensate (15) in patients with pneumonia, which had no correlation to serum levels of HGF, indicated a local production of HGF during pneumonia. Furthermore we studied the stability of HGF in serum (16) as well as in faeces (unpublished data) and found that HGF was very stable in samples and several freeze-thaw cycles; different buffers or several years of storage at $-20^{\circ}C$ did not affect faeces HGF concentrations significantly.

The aim of the present study was to investigate the local and systemic production of HGF during acute gastroenteritis, its correlation to some other inflammatory indicators such as white blood cell count (WBC) and C-reactive protein (CRP), and its implication as a diagnostic marker. Therefore, paired HGF concentrations in serum and faeces of patients with infectious gastroenteritis were compared to patients with non-infectious diarrhoea due to other diseases (patient controls) as well as to healthy controls.

MATERIAL AND METHODS

Patients

Between 2000 and 2001, 54 consecutive patients admitted to the Department of Infectious Diseases in Linköping with signs of acute untreated infectious diarrhoea were asked to join the study. Blood and faeces samples were taken at admittance. Ordinary blood tests for white blood cell count (WBC) and C-reactive protein (CRP) were conducted for all patients. Samples from faeces were cultured

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(Campylobacter, Salmonella and Shigella) for all patients, and laboratory diagnostic tests for Rotavirus (antigen detection) and Calicivirus (electron microscopy) were also conducted for all cases. Both cultures and cytotoxin analysis for Clostridium difficile were conducted for all patients. The samples were cultured for enterohemorrhagic Escherichia coli (EHEC) for cases of bloody diarrhoea. Direct microscopic analysis of faeces for detection of cysts and helminths was carried out for all patients who had been abroad. Cultures and cytotoxin analysis (toxin A) were positive for Clostridium difficile in 11 cases. Cultures revealed growth of Campylobacter jejuni in 13 cases. Salmonella art (Salmonella. DO) was found in 6 cases (Group 1). One patient had Blastocystis hominis in faeces. Two patients had gastroenteritis caused by Rotavirus. Faeces (but not blood) samples from a further 8 patients with Rotavirus, stored at -70°C at the Department of Virology, were analysed retrospectively (unidentified, no further information available) for the presence of HGF. This gave a total of 10 samples of Rotavirus (Group 2).

Cultures and viral diagnostic tests were negative in 21 cases. In 10 of these cases, non-specific diarrhoea due to other diseases was found (non-infectious acute diarrhoea, Group 3: peritonitis in 2, partial intestine obstruction in 1, lactose intolerance in 1, overconsumption of laxantia in 1, side effects after initiating piperacillin-tazobactam and penicillin in 2, short episode of diarrhoea during pneumonia in 2, fever of unknown origin with a short episode of diarrhoea in 1 case). In a further 8 of these 21 cases, infectious diarrhoea was highly suspected on clinical grounds in spite of negative cultures (fever, vomiting, diarrhoea and/or history of travelling abroad to endemic areas and/or other causes of diarrhoea in the environment). These cases are considered as probable infectious gastroenteritis (Group 5). In the remaining 3 patients, no clear cause of diarrhoea was found (unclear diarrhoea, Group 6): an infectious genesis could be neither ruled out nor accepted (slightly increased white blood cell count, diarrhoea, vomiting and acute renal failure in 2 cases; slightly increased CRP, positive faeces haemoglobin, diarrhoea and vomiting in 1 case) (Table I). Faeces ($n = 26$) and blood samples ($n = 25$) were taken and stored from patients who attended a follow-up visit at 4 weeks (6 cases with Clostridium difficile, 7 cases with Campylobacter jejuni, 3 cases with Salmonella, 2 cases of Rotavirus and 8 cases with probable infectious gastroenteritis).

Healthy controls

Serum and faeces samples were taken from 11 healthy vaccination volunteers without signs of infection or diarrhoea (Group 4). Faeces culture and diagnostic tests for Rotavirus and Calicivirus were negative in all of these cases.

Determination of HGF in serum

A commercially available ELISA kit (Quantikine HGF immunoassay, R&D Systems Inc., Minneapolis, USA) was used to determine immunoreactive HGF. The method is a solid phase ELISA. The serum samples, stored at -70°C, were centrifuged at 1000 G for 15 min prior to analysis. Double measurements of HGF in serum were made to assess the methodological error. The methodological error was calculated by Dahlberg's equation and found to be 6.7% (coefficient of variation).

Determination of HGF in faeces

The faeces samples (5–20 g) were stored at -70°C pending analysis. Prior to analysis, they were freeze-dried and dissolved in distilled water to yield a concentration of 0.2 g faeces/ml. A commercially available ELISA kit (Quantikine HGF immunoassay, R&D Systems Inc., Minneapolis, USA) was used to determine immunoreactive HGF. The method was modified according to the manufacturer's specifications to determine HGF in cell culture supernate samples. The methodological error was 15% (coefficient of variation).

Western immunoblots

Proteins from faeces were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in 12% acrylamide gels (8×5.5 cm) and transferred to a cylindrical polyvinylidene fluoride (PVDF) membrane with electroblotting. The membrane was first incubated with primary goat antibodies against human HGF (1:250, R&D Systems), then with secondary rabbit anti-goat HRP-labelled antibodies (1:40 000, BioRad Laboratories) and finally visualised by enhanced chemiluminescence (Amersham Bioscience) onto an X-ray film.

Statistical analysis

Serum and faeces HGF values were log-normally distributed, and analyses of variance by groups (ANOVA, followed by Duncan's test in the case of significance) and linear regression were therefore used after transforming the values into their natural logarithms. A probability level of $p < 0.05$ was considered statistically significant. The different patient groups included in the ANOVA statistics are shown in Table I. After the primary analyses showing no differences between the 3 bacterial infections for either serum or faeces HGF concentrations, these groups (30 patients) were combined (Group 1). As infection with amoebae represented a single case only, it has been omitted from the ANOVA statistics.

RESULTS

HGF concentration in faeces

There were no significant differences in faeces HGF concentrations in the patients with gastroenteritis caused

Table I. *Patient characteristics and HGF concentrations in serum and faeces at the acute stage of diarrhoea. nd = non-defined*

Causes of diarrhoea	Number of samples Faeces/serum	Median age in years (range)	Female/ Male	Mean serum HGF ng/ml (range)	Mean faeces HGF ng/g (range)
Bacteria	30/24	45 (17–93)	15/15	1.75 (0.8–3.39)	128 (41.5–580)
Virus	10/2	nd	nd	1.37 (0.98–1.92)	35 (5.5–69)
Probable infection	8/8	64 (28–84)	4/4	2.39 (1.00–6.47)	69 (36–154)
Amoebae	1/1	53	1/0	1.48	47
Unclear diarrhoea	3/3	78 (72–83)	1/2	1.36 (1.22–1.50)	38 (30–62)
Non-infectious diarrhoea (patient controls)	10/7	71 (41–87)	6/4	1.90 (0.74–6.48)	6.5 (1.77–20)
Healthy controls	11/11	36 (16–66)	8/3	0.64 (0.48–1.28)	2.4 (0.58–9.8)

by Clostridium difficile, Campylobacter jejuni or Salmonella (Table I). These 3 are therefore treated as a single group (bacterial infections) in the following analyses. Using ANOVA, 4 statistically significantly different groups could be identified: 1) bacterial infections with high concentrations, 2) viral infections with moderate concentrations, 3) non-infectious acute diarrhoea with an average level of HGF slightly above the normal range (patient controls) and 4) normal controls with normal concentrations. There was a complete separation (on an individual basis) between Group 1 on the one hand and Groups 3 and 4 on the other with a cut-off value of around 40 ng/g faeces, with the viral infections between these groupings (Fig. 1). Probable infection (Group 5) had high concentrations close to Group 1 but was statistically not significantly different from the moderate concentration group (viral infections), to which unclear diarrhoea (Group 6) and the 1 patient with amoebae infection also belonged. Using a cut-off concentration of 20 ng/g, the overall sensitivity of faeces HGF to distinguish infectious gastroenteritis (bacterial, viral, probable infection) was 98% with a specificity of 100%.

Patients with acute infectious gastroenteritis returned to normal faeces HGF concentrations at convalescence and this reduction was statistically significant ($p = 0.0001$). There were no significant correlations between age, the length of disease after onset of diarrhoea and faeces HGF concentrations. There was no significant correlation between CRP, WBC and faeces HGF concentrations (data not shown).

HGF concentration in serum

Serum HGF was significantly lower in normal controls (ANOVA, $p < 0.0001$) compared to any other group, but there were no significant differences between the other groups ($n = 35$, Table I). The serum HGF concentrations decreased significantly at convalescence (4–6 weeks, $n = 25$, median 0.86 ng/ml, $p < 0.001$). There were no significant differences between serum HGF concentrations in the healthy controls and levels in the patients with infectious gastroenteritis at convalescence.

Interrelationship between serum HGF and faeces HGF

Higher levels of HGF were found in faeces compared to serum in all of the cases. When excluding healthy controls, there was no significant correlation between HGF concentrations in faeces and serum ($r = 0.09$, $p = 0.5$).

Western immunoblots

Two bands corresponding to the α- and β-chain of HGF were detected with apparent molecular masses of 55 and 25 kDa respectively (Fig. 2).

DISCUSSION

The gastrointestinal mucosa has a remarkable ability to repair damage, and growth factors play an important role in the regeneration of injured cells in gastrointestinal organs (17). The effects of cytokines, epidermal growth factor (EGF) (18), transforming growth factor-α (TGF-α) (19) and fibroblast growth factor (FGF) (20) during gastrointestinal injuries have been investigated. HGF is the most potent mitogen for hepatocytes as well as many other epithelial cell types (6). Nishimura et al. (1998) (21) showed that HGF was the most potent of the cytokines (TGF-α, TGF-β and keratinocyte growth factor) in accelerating repair of the damaged monolayer of an epithelial cell line derived from normal rat small intestine. An overexpression of HGF and its receptor c-met was observed in the inflamed mucosa of ulcerative colitis by Kitamura et al. (2000) (22). Nusrat et al. (1994) (23) suggested that HGF could influence the intestinal epithelia in a paracrine fashion resulting in a dramatic increase in the rate of epithelial wound closure.

At our centre, patients with acute diarrhoea are offered isolated care at the Department of Infectious Diseases until the faeces culture results are shown to be negative. In the

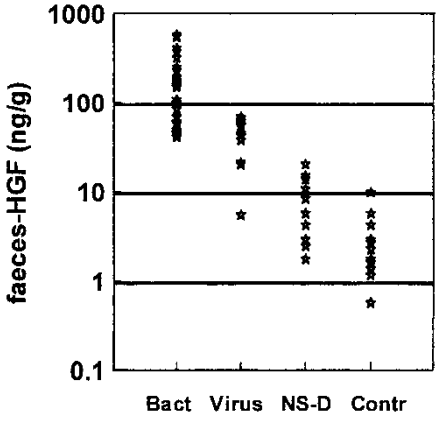

Fig. 1. Fig. 1 shows the individual levels of faeces HGF in different groups: Bact = bacterial gastroenteritis, Virus = viral gastroenteritis, NS-D = non-infectious acute diarrhoea, Contr = healthy control group.

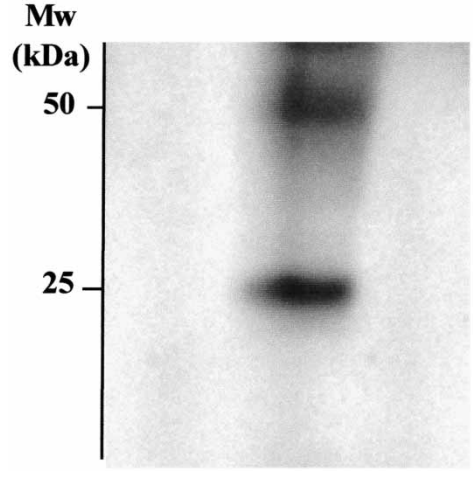

Fig. 2. Demonstration of HGF (α- and β-chain) in faeces of a patient with Salmonella enteritis with Western immunoblot.

present prospective study, we recruited patients with acute diarrhoea consecutively and therefore had access to 2 different diagnostic groups: patients with verified faeces culture or virus diagnostic tests or a high suspicion of infectious diarrhoea, and a group of patients where an infectious genesis of the diarrhoea was subsequently ruled out (non-infectious acute diarrhoea). The third group was the healthy control group without diarrhoea. Although serum HGF concentrations could not distinguish between the first two groups, though both might have had high serum HGF concentrations, we found that faeces HGF concentrations were dramatically higher in those patients where diarrhoea was caused by infection than in the patients with non-infectious acute diarrhoea. The lack of correlation between serum HGF and faeces HGF concentrations during gastroenteritis might indicate a local production of HGF at the injured area of intestine.

Viral gastroenteritis can cause outbreaks of diarrhoea, and there are still no reliable markers that can distinguish transmittable gastroenteritis from other causes of diarrhoea. CRP levels rise during bacterial infections, but levels are low during viral disease (24). However, we found that in contrast to serum HGF concentrations or CRP, faeces HGF concentrations were high during viral gastroenteritis, possibly due to the presence of mucosal inflammation and injury caused by infection.

Matsuno et al. (1997) (25) showed that HGF was present around the neutrophils, infiltrating into the lamina propria, which was biopsied from endoscopically active colonic mucosa in patients with ulcerative colitis. Such a phenomenon might be responsible for the high production and release of HGF in faeces during acute infectious gastroenteritis.

Faeces HGF concentrations decreased rapidly over time and in 3 cases of chronic Salmonella carriers (not included in the patient material) the faeces HGF concentrations were low. In the patients with diarrhoea caused by Clostridium difficile that were treated by antibiotics (not included in patient material), the levels of faeces HGF decreased dramatically after treatment (data not shown).

In addition to the ELISA method, the presence of high amounts of HGF in faeces was confirmed by Western immunoblots. Two typical chains were detected which might show the presence of HGF in faeces (6, 8, 9) (Fig. 2). The bands were detectable in infectious gastroenteritis, but no bands were found in the faeces samples from acute non-infectious gastroenteritis, normal control or faeces of patients with infectious gastroenteritis at convalescence (data not shown).

We conclude that determination of faeces HGF concentrations during acute gastroenteritis might have an implication in distinguishing transmittable diarrhoea requiring isolation from other causes of diarrhoea, which could eventually result in considerable savings of resources. It might also be of value in identifying the cause of diarrhoea and early initiation of appropriate therapy in cases of acute non-infectious gastroenteritis where therapy might otherwise be postponed until the results of traditional microbiological tests were available.

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