

Keratinocyte growth factor and coeliac disease

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Accepted for publication 26 February 2001

Abstract

Background—Coeliac disease is characterised by increased epithelial renewal associated with a mucosal T cell response to gliadin. Keratinocyte growth factor (KGF) is produced by cytokine activated gut stromal cells and may be a link between mucosal T cell activation in untreated coeliac disease and epithelial hyperplasia.

Aims—To characterise expression of KGF in coeliac disease.

Methods—KGF transcripts in coeliac disease were measured by quantitative competitive reverse transcription-polymerase chain reaction (RT-PCR) and localised using *in situ* hybridisation. KGF production by gluten reactive CD4+ T cell clones was examined. In addition, KGF transcripts were measured following *ex vivo* challenge of coeliac biopsies with a peptic-tryptic digest of gliadin.

Results—KGF transcripts were elevated in coeliac biopsies compared with normal controls but were not different from non-coeliac disease controls. By *in situ* hybridisation, KGF mRNA containing cells were present in the upper half of the lamina propria, most abundantly just under the epithelium. There was no signal from cells within the epithelium. Gluten reactive T cell clones did not make KGF. *In vitro* challenge of coeliac biopsies generated a strong interferon γ response but a specific KGF response could not be detected because of an extremely high number of KGF transcripts in all cultured biopsies.

Conclusions—KGF is overexpressed in coeliac biopsies and in tissues with non-coeliac enteropathy. No evidence was found for KGF production by intraepithelial lymphocytes or lamina propria T cells. (Gut 2001;49:176–181)

Keywords: coeliac disease; keratinocyte growth factor; mRNA expression

In untreated coeliac disease (CD), transformation of the small intestinal mucosa is one of the most striking examples of immune mediated enteropathy. The characteristic villus atrophy and crypt hyperplasia is in fact mucosal remodelling and tissue growth, in this case expansion of the lamina propria volume with a reduction in epithelial surface area.¹ The exact sequence of events that leads to the flat mucosa is not known. It has been suggested that immune mediated epithelial damage leads to compensatory crypt hyperplasia² in which the epithelium causes the transformation. On the

other hand, expansion of the lamina propria compartment could also be responsible for crypt hypertrophy with unknown events also leading to increased epithelial division.

Normal epithelium renewal is controlled largely by transforming growth factor α , epidermal growth factor, and transforming growth factor β working in an autocrine fashion within the epithelium.³ Subepithelial myofibroblasts and basement membranes are essential for epithelial formation but whether they play a role in controlling basal levels of proliferation is not known. An attractive notion to explain the hyperplasia seen in all forms of gut inflammation is that locally released cytokines increase the production of growth factors from lamina propria stromal cells, which are then mitogenic for epithelial cells. For example, in the stomach, interleukin 1 induced overexpression of hepatocyte growth factor may drive increased epithelial renewal.⁴ In the small bowel and colon, there has been much interest in the role of keratinocyte growth factor (KGF, FGF-7). KGF is a well characterised epithelial mitogen and is produced by cytokine activated mesenchymal cells.⁵ Injection of recombinant KGF into rats increases gut epithelial proliferation.⁶ KGF transcripts are abundantly expressed in the mucosa in inflammatory bowel disease (IBD).^{7–8} In addition, in a model system of T cell mediated crypt hyperplasia, we have shown directly that KGF is involved in increasing epithelial proliferation.⁹ While the majority of evidence suggests that in the gut KGF is made by stromal cells, there is evidence that in mice epithelial $\gamma\delta$ T cells can also make KGF.¹⁰ Thus in the case of CD, where the increase in intraepithelial (IEL) $\gamma\delta$ T cells is well documented, it is highly relevant to determine if KGF is also expressed by these cells.^{11–12}

In this study therefore we have analysed KGF in CD to determine if it is overexpressed and the location of KGF mRNA containing cells. In addition, we have attempted to determine if KGF is made by T cells. Finally, we carried out experiments to determine if there was induction of excess KGF following gluten challenge *ex vivo*.

Patients and methods

PATIENTS

Twenty five patients with active CD, five patients with non-coeliac enteropathy (NCE), and 16 age matched normal controls referred

Abbreviations used in this paper: CD, coeliac disease; KGF (FGF-7), keratinocyte growth factor; IEL, intraepithelial lymphocytes; NCE, non-coeliac enteropathy; RT-PCR, reverse transcription-polymerase chain reaction; IFN- γ , interferon γ ; IBD, inflammatory bowel disease; LPL, lamina propria T cells.

to the Department of Paediatric Gastroenterology and Endoscopy, University Federico II, Naples, for abdominal complaints, growth failure, weight loss, diarrhoea, or vomiting were enrolled. Biopsies from the proximal jejunum were taken by gastroduodenal endoscopy, quickly snap frozen in liquid nitrogen, and stored at -80°C for quantitative reverse-transcription polymerase chain reaction (RT-PCR) analysis. CD patients (15 females, 10 males) ranged in age from 0.7 to 13.4 years (median 3), patients with NCE (two females, three males) ranged in age from 0.9 to 16 years (median 4), and normal control patients (five females, 11 males) ranged in age from 1.6 to 16.9 years (median 7). The diagnosis of CD was confirmed by ESPGHAN criteria including flattening of villi and increased number of IELs in the initial biopsy, and a prompt clinical response to a gluten free diet.¹³ Normal control patients had no significant abnormality of the small intestinal mucosa and were mainly affected by oesophagitis (12/16), gastritis (3/16), or irritable bowel syndrome (1/16). Patients with NCE were affected by gastritis and/or duodenitis (2/5), IgA deficiency and cow's milk intolerance (1/5), atopic dermatitis (1/5), or food intolerance (1/5). The proximal jejunal mucosa showed a patchy lesion with moderate-low grade atrophy in three of five patients, including the two patients with gastritis and duodenitis and the patient affected by food intolerance. By immunohistochemistry in 2/5 patients, both intraepithelial CD3+ and lamina propria CD25+ cells were increased. In 1/5 patients only intraepithelial CD3+ cells and in 2/5 only lamina propria CD25+ cells were increased. None showed increased intraepithelial $\gamma\delta$ + T cells and thus it is unlikely they had latent CD.¹²

For ex vivo challenge studies, six adult CD patients (two men, four women) aged 15–44 years (median 30.5) diagnosed on the basis of ESPGHAN criteria and eight normal controls (four men, four women) aged 19–60 years (median 39) investigated for upper abdominal complaints were enrolled. Five CD patients were on a gluten free diet for a median period of eight years (range 1–12) while one patient confirmed low compliance with this diet. All patients were recruited after appropriate local ethics committee approval and informed consent was obtained. Jejunal mucosa was histologically normal in both groups except for the untreated CD patient with atrophy, crypt hyperplasia, and increased intraepithelial lymphocyte infiltration. In the control group, 3/8 subjects were positive for gastritis and *Helicobacter pylori*. From each patient four biopsy specimens from the proximal jejunum were obtained by gastroduodenal endoscopy and placed immediately in ice chilled saline solution and processed within 30 minutes for in vitro organ culture experiments.

METHODS

In vitro organ culture experiments

Mucosal specimens were cultured principally as described elsewhere.¹⁴ Briefly, biopsies were placed on a stainless steel mesh with the

mucosal face upwards in the central well of an organ culture dish in Trowell (6.5 ml)/NCTC-135 (2.5 ml) medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 $\mu\text{g}/\text{ml}$) (Life Technologies-Gibco BRL, Milan, Italy) in the presence or absence of a peptic-tryptic digest (Frazer III fraction) of gliadin (Sigma, Milan, Italy) at 1 mg/ml. The dishes were placed in a sterile anaerobic jar which was gassed with 95% O_2 /5% CO_2 and incubated at 37°C . Biopsies at time 0 (baseline) and after eight hours of culture were snap frozen and stored at -80°C for RT-PCR analysis.

RNA standards for quantitative RT-PCR

Plasmids pHCQ1 (provided by Dr MF Kagnoff, Department of Medicine, University of California, San Diego, USA) and pMBEK encoding the sequence of interferon γ (IFN- γ) and KGF, respectively, were used for quantitative RT-PCR, as described previously.^{8, 15} To generate standard RNA molecules, plasmids were linearised with *HindIII* and transcribed in vitro using T7 RNA polymerase under conditions recommended by the supplier (Promega, Biotech, Milan, Italy).

RNA isolation and competitive quantitative RT-PCR

Total tissue RNA was extracted from whole mucosal jejunal biopsies using the Trizol reagent (Gibco Life Technologies, Milan, Italy) according to the manufacturer's instructions and quantified at 260 nm. The integrity of the RNA was checked by agarose gel electrophoresis. A constant amount of total RNA (1 μg) and serial 10-fold dilutions of standard RNA molecules (1 pg to 0.1 fg) were co-transcribed into complementary cDNA in a 20 μl reaction mixture containing 50 mM Tris, pH 8.3, 75 mM KCl, 3 mM MgCl_2 , 500 μM each of dATP, dCTP, dTTP, and dGTP (Pharmacia Biotech, Milan, Italy), 10 mM DTT, 0.5 μg oligo (dT)_{12–18} (Pharmacia Biotech) and 100 U M-MLVRT (Gibco Life Technologies). PCR was performed with 5 μl of cDNA in 50 μl of 20 mM Tris HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl_2 (for KGF), or 2 mM MgCl_2 (for IFN- γ), 200 μM each of dATP, dCTP, dTTP, and dGTP, and 30 pM of 5' and 3' primers. PCR primers, amplification conditions, and molecular weights of standard and target molecules have been described elsewhere.^{8, 15} PCR products were visualised on a 2% agarose gel using Vista-Green dye (Amersham International, Milan, Italy). Densitometric analysis of fluorescent bands was carried out on a STORM-860 analyser using ImageQuant software (Molecular Dynamics, Sunnyvale, California, USA). The ratio of standard/target RNA was plotted against the starting number of standard RNA molecules on a double logarithmic scale to determine the copy number in each sample. The data were presented as number of transcripts/ μg total RNA.

Gluten challenge of mucosal CD4+ T cell clones

Gut derived HLA-DQ restricted and gluten reactive T cell clones and lines were generated as detailed elsewhere.¹⁶ Two different gluten

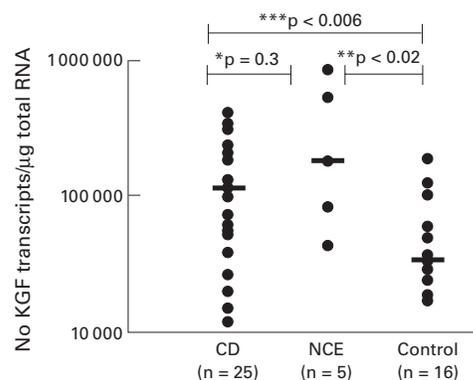


Figure 1 Keratinocyte growth factor (KGF) mRNA transcripts in whole small intestinal biopsies from patients with coeliac disease (CD), non-coeliac enteropathy (NCE), and normal controls. Bars represent median KGF mRNA transcripts. ***CD versus controls, $p < 0.006$; **NCE versus controls, $p < 0.02$; *CD versus NCE, $p = 0.3$

reactive CD4+ T cell clones and two CD4+ T cell lines were challenged in vitro with phorbol myristate acetate (10^{-8} M) plus immobilised anti-CD3 antibodies (10 $\mu\text{g}/\text{ml}$) coated on Dynabeads M450 (Dyna, Oslo, Norway) or with a peptic-tryptic digest of gliadin at 3 mg/ml. Epstein-Barr virus transformed B cell lines were used as antigen presenting cells. For each experiment, 1×10^5 Epstein-Barr virus transformed B cells were incubated with 1×10^5 T cells in RPMI medium supplemented with

15% human serum and after 0, 2, 4, 8, and 18 hours T cells were collected, snap frozen in liquid nitrogen, and stored at -80°C for RT-PCR analysis.

In situ hybridisation

A 410 bp Xba/Pst1 fragment (nucleotides 510–920) of human KGF was amplified by RT-PCR from a 16 week fetal small intestine sample and subcloned into pGEM-3Zf(-) plasmid (Promega, Southampton, UK) at the corresponding restriction sites and the sequence of the probe was confirmed by double stranded dideoxysequencing (Amersham Pharmacia, St Albans, UK). Sense and antisense RNA probes were prepared by linearising the plasmid with BamH1 and HINDIII enzymes, respectively, followed by in vitro transcription. In situ hybridisation was performed on six control and six untreated CD formalin fixed paraffin embedded tissue samples. After overnight hybridisation at 55°C , the slides were appropriately washed, dehydrated, and dipped in Ilford 5 emulsion for autoradiography, as described previously.⁸ As controls, parallel sections were hybridised to equal radioactive amounts of antisense β -actin riboprobe.

Statistical analysis

Statistical analysis was performed using non-parametric tests: Mann-Whitney U for two

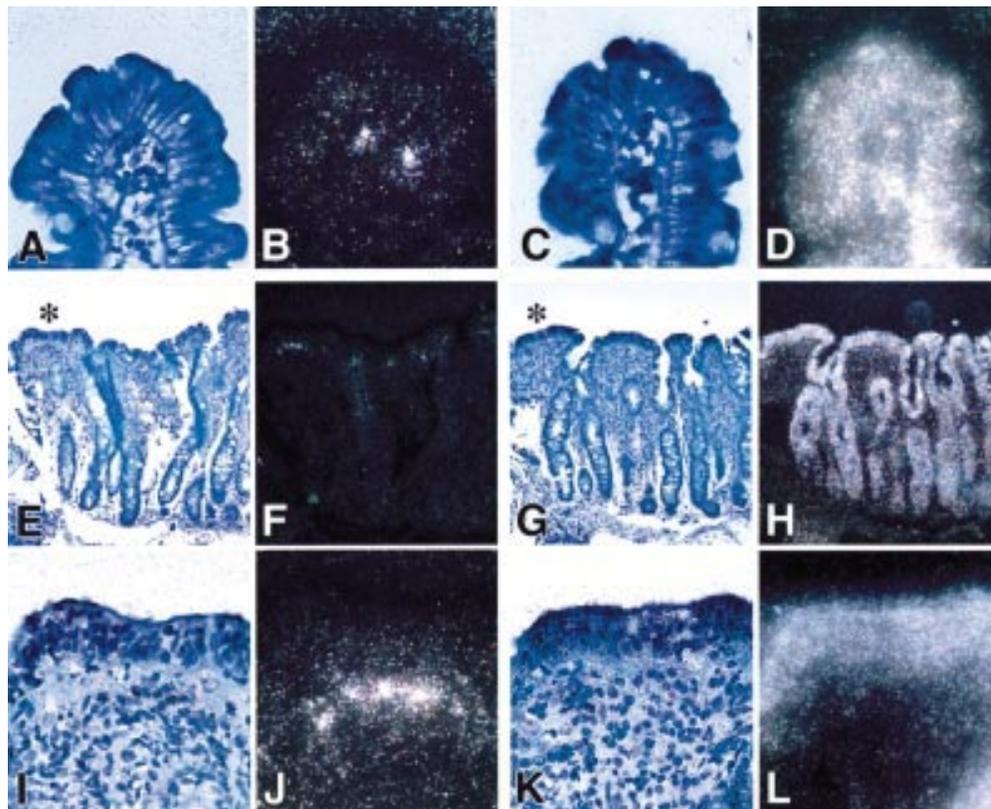


Figure 2 Detection of keratinocyte growth factor (KGF) mRNA by in situ hybridisation. (A, B) and (C, D) represent light/dark images of a control section hybridised with antisense probes to KGF mRNA (A, B) and to β -actin (C, D) as a positive control at a magnification of 50 \times . Very few KGF transcripts were detectable in control sections below the tip of the villi. (E) and (F) show light and dark field images, respectively, at 20 \times magnification stained for KGF. In (I) and (J) at 50 \times magnification the flat villi indicated by an asterisk in (E) are shown in more detail. KGF positive hybridising cells are distributed in the subepithelial region of the lamina propria below the tip of the flattened villi. (G, H) (20 \times) and (K, L) (50 \times) represent light (G, K) and dark field (H, L) images of the corresponding β -actin staining as a positive control.

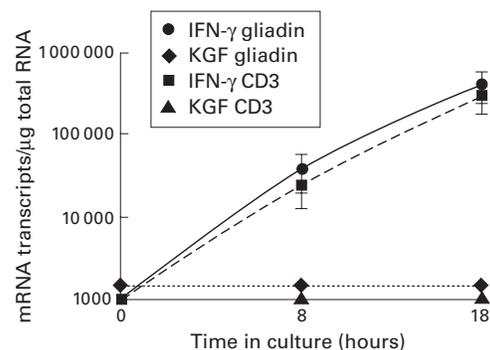


Figure 3 Number of keratinocyte growth factor (KGF) and interferon γ (IFN- γ) mRNA transcripts in four gut derived gluten reactive CD4⁺ T cell clones and lines challenged in vitro with a peptic-tryptic digest of gliadin or anti-CD3 antibodies for eight and 18 hours. Each point at the indicated time represents mean (SEM) values. KGF mRNA expression was below the detection limit (1000 transcripts/ μ g total RNA) while IFN- γ mRNA transcripts were increased after eight and 18 hours in the presence of gliadin or anti-CD3 antibodies (positive control).

independent samples and Wilcoxon signed rank test for paired samples.

Results

ANALYSIS OF KGF GENE EXPRESSION IN WHOLE BIOPSIES FROM CD PATIENTS

The abundance of KGF mRNA transcripts in control tissue was low (median 33 105 transcripts/ μ g total RNA; range 2592–181 602). In untreated CD biopsies, KGF transcripts were markedly increased (median 112 901 transcripts/ μ g total RNA; range 12 273–404 570; $p < 0.006$). In NCE, KGF mRNA transcripts were also abundant (median 177 025; 95% confidence interval 42 956–831 332; $p < 0.02$) and at a level not significantly different from that in CD patients ($p = 0.3$) (fig 1). As an indication of ongoing inflammation, IFN- γ mRNA transcripts were also measured and these were significantly upregulated in CD compared with normal and non-coeliac inflamed mucosa ($p < 0.0001$) (data not shown), confirming previous studies by Nilsen and coworkers.¹⁷

IN SITU HYBRIDISATION ANALYSIS

In sections of control small bowel, KGF mRNA was barely detectable with only few positive hybridising cells at the top of occasional villi (fig 2B, dark field image). In contrast, in coeliac mucosa, KGF mRNA positive cells were distributed in the lamina propria in the subepithelial region of the flattened villi (fig 2F, dark field image at 20 \times and fig 2J at 50 \times magnification). No transcripts were detected in the pericryptal stroma. In particular, no KGF positive hybridising cells were seen within the epithelium. Sections hybridised with an antisense probe for β -actin as a positive control showed abundantly positive cells throughout the tissue in coeliacs (fig 2H, L) and controls (fig 2D). In previous studies, a sense KGF probe gave no specific pattern of hybridisation.^{8,9}

ANALYSIS OF KGF AND IFN- γ GENE EXPRESSION IN MUCOSAL CD4⁺ T CELL CLONES

Gluten reactive CD4⁺ T cell clones and lines obtained from coeliac small intestinal lamina

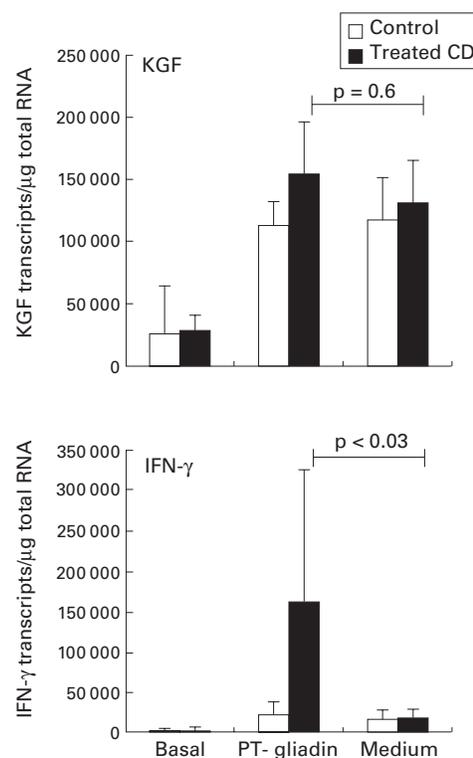


Figure 4 Keratinocyte growth factor (KGF) and interferon γ (IFN- γ) mRNA transcripts measured by competitive quantitative RT-PCR in treated coeliac disease (CD) ($n = 6$) and control biopsies ($n = 8$) challenged in vitro with gliadin. Small intestinal biopsies were cultured for eight hours in the presence or absence of a peptic-tryptic (PT) digest of gliadin (1 mg/ml). Bars represent median (SEM). IFN- γ mRNA transcripts were markedly induced by gliadin in coeliac mucosa compared with biopsies cultured in medium alone ($p < 0.03$). KGF mRNA transcripts were increased in both coeliacs and controls, in the presence and absence of gliadin.

propria were challenged in vitro with a peptic-tryptic digest of gliadin or anti-CD3 antibodies. At two and four hours, IFN- γ mRNA transcripts were not detectable (data not shown). However, a marked induction in IFN- γ mRNA transcripts was observed at eight and 18 hours, while KGF transcripts were below the limit of detection (fig 3).

ANALYSIS OF KGF AND IFN- γ GENE EXPRESSION IN CHALLENGED CD BIOPSIES

In treated coeliac biopsies challenged in vitro with gliadin, the number of IFN- γ mRNA transcripts was markedly upregulated compared with biopsies cultured in medium alone ($p < 0.03$) or with control biopsies challenged with gliadin. KGF mRNA transcripts were increased in both coeliac and control cultures compared with baseline levels, regardless of the presence of gliadin (fig 4).

Discussion

CD is characterised by small intestinal mucosal damage and nutrient malabsorption following dietary ingestion of prolamines in wheat, rye, and barley in genetically susceptible individuals. One of the hallmarks of the morphological features of the coeliac lesion is the atrophy of villi, accompanied by crypt hyperplasia and marked infiltration in the

epithelium of T cells.¹² Migration of T cells into the villous epithelium is the first immunopathological feature observed in jejunal mucosa of treated coeliac patients on gluten challenge.¹⁸ Gluten activated lamina propria T cells and culture explants from coeliac patients can produce proinflammatory cytokines of the Th1 type, dominated by IFN- γ .¹⁷ In human fetal gut explants, experimental activation of T cells by anti-CD3 antibodies or polyclonal activators results in villus atrophy, crypt hyperplasia, and intraepithelial infiltration of lymphocytes, morphological features very similar to CD.^{2, 19} In human diseases, such as IBD and graft versus host disease, T cell mediated inflammation is followed by crypt hyperplasia.²⁰ These data suggest that T cell activation drives crypt hyperplasia.

This study was undertaken to define the possible role of KGF in mediating crypt hyperplasia in CD and furthermore to clarify the cellular basis of KGF transcripts in CD. By competitive RT-PCR we found a marked increase in the number of KGF mRNA transcripts in both coeliac and non-coeliac enteropathy compared with control non-inflamed mucosa. Our data are in line with other studies on gastrointestinal diseases, such as IBD, where the presence of inflammation and increased epithelial infiltration showed a parallel upregulation of this growth factor.^{7, 8}

Another important point addressed in our study was the cellular source of KGF transcripts in CD. We and others have previously shown that mesenchymal stromal cells of the lamina propria are a major source of KGF in IBD.^{7, 8} In human fetal gut organ culture, where stimulation with bacterial superantigens drives T cell activation and morphological features very similar to CD, KGF is also overexpressed by mesenchymal stromal cells.⁹ Nevertheless, in mice, activated $\gamma\delta$ T cells of the skin and from the gut have been reported to make KGF, suggesting that IEL derived KGF may contribute to epithelial cell proliferation.¹⁰ In humans, expression of KGF by IELs remains unclear. Isolated $\gamma\delta$ IELs from human colonic inflamed mucosa have been reported to express KGF.²¹ However, the signal observed by ribonuclease protection assay was weak and was not seen in all patients. There is the possibility that the weak signal was due to a few contaminating fibroblasts which express KGF mRNA in abundance.^{8, 9} As intraepithelial infiltration of $\gamma\delta$ IELs is a hallmark of the CD lesion, we investigated expression and localisation of KGF mRNA by in situ hybridisation. Interestingly, an increased number of positive cells were present but were confined to the lamina propria immediately below the mucosal epithelial layer of the flattened villi. No KGF producing cells were seen within the epithelium, despite an intense infiltrate of intraepithelial lymphoid cells. Our data thus indicate that intraepithelial $\gamma\delta$ T cells of the gut mucosa do not produce KGF, at least not in levels detectable by in situ hybridisation, and are not the principal source of KGF mRNA in vivo. To

extend these findings, we also analysed activated lamina propria T cells (LPLs) for their capacity to synthesise KGF. CD4+ gluten reactive T cell clones and lines were isolated from the mucosa of coeliac patients and activated in vitro by anti-CD3 antibodies or a peptic-tryptic digest of gliadin. A time dependent increase in IFN- γ mRNA was observed with no corresponding increase in KGF expression, suggesting that LPLs do not contribute to KGF expression in CD. The present data are consistent with previous observations in IBD where KGF expression in the lamina propria was undetectable by immune cells.^{8, 21} Similarly, in our study there was no correspondence between the in situ hybridisation signal for KGF and localisation of T cells in the lamina propria. Our work thus indicates that overexpression of KGF in coeliac mucosa is not derived from T cells (LPL and IELs).

As observed in human IBD and in the fetal gut, high levels of KGF in coeliac mucosa may be produced by mesenchymal stromal cells.^{8, 9} It has been suggested that immune mediated epithelial damage may lead to compensatory crypt hyperplasia² but on the other hand it is also possible that T cell mediated chronic inflammation of the mucosa leads to activation of cytokines and growth factors responsible for mucosal remodelling. In fact, we had anticipated that localisation of KGF transcripts would be similar to Crohn's disease and ulcerative colitis—that is, throughout the lamina propria—and it was a surprise that they were located below the epithelium. However, other data suggest that the subepithelial layer is a site of tissue remodelling in active CD. Increases in MMP-1, MMP-3, and TIMP-1 mRNA expression in CD have been observed in fibroblasts and macrophages in the stroma underneath the surface epithelium and not in the pericryptal stroma.²²

As in vivo challenge with gluten of treated coeliac patients is difficult, we attempted to study the early events induced by gluten in the coeliac treated mucosa by ex vivo organ culture. In fact, it may be that expression of KGF during the evolution of the lesion is different from the steady state in flattened mucosa. Previous studies have shown that in vitro challenge with gluten of treated CD biopsy specimens leads to activation of lamina propria T cells and macrophages, induces intraepithelial infiltration of lymphocytes, and expression on crypt epithelial cells of HLA-DR.²³ We therefore challenged treated coeliac biopsies ex vivo with a peptic-tryptic digest of gluten and analysed mRNA expression for IFN- γ and KGF after eight hours. High levels of IFN- γ mRNA transcripts induced by gliadin were seen in coeliac biopsies as evidence of immunological activation. However, KGF transcripts were overexpressed in both control and coeliac biopsies cultured without or in the presence of gliadin compared with baseline levels snap frozen within 30 minutes after collecting the sample. This marked overexpression is probably a response to injury, as the biopsy is torn from the mucosa.

Another interesting result was the finding that the abundance of KGF mRNA was also increased substantially in the NCE group. The molecular causes responsible for the mucosal changes were not known although there was some evidence of T cell activation in these patients (see methods). This suggests that KGF is involved in epithelial proliferation driven by T cell mediated inflammation, regardless of the stimulus. This is a very similar situation to that of tumour necrosis factor α in inflamed colonic mucosa where tumour necrosis factor α secreting cells are increased, regardless of the cause of the inflammation.²⁴

In conclusion, in CD, as in other forms of gut inflammation characterised by crypt hyperplasia, KGF mRNA is overexpressed. Our data indicate that KGF is not produced by lamina propria T cells or intraepithelial $\gamma\delta$ T cells. It is likely that in CD, KGF expression is induced by proinflammatory cytokines in a paracrine and/or autocrine fashion and is involved in mucosal remodelling and crypt hyperplasia.

This study was carried out with financial support from the Commission of the European Communities, specific RTD programme "Quality of Life and Management of Living Resources", QLK1-CT-1999-00037, Evaluation of the prevalence of CD and its genetic components in the European population. It does not necessarily reflect its views and in no way anticipates the Commission's future policy in this area. The work was also partly supported by "Istituto Superiore di Sanita': Sorveglianza degli effetti nocivi da alimenti: intolleranza ai cereali (Convenzione 98/JK/T3)" and "MURST-PRIN: Citochine e patogenesi del danno mucosale nella malattia coeliaca", by the Wellcome Trust, the special trustees of St Bartholomew's Hospital, and the Crohn's in Childhood Research Association.

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