Human keratin 8 mutations that disturb filament assembly observed in inflammatory bowel disease patients

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Accepted 5 December 2003
Journal of Cell Science 117, 1989-1999 Published by The Company of Biologists 2004
doi:10.1242/jcs.01043

Summary

We have identified miss-sense mutations in keratin 8 in a subset of patients with inflammatory bowel disease (Crohn disease and ulcerative colitis). Inflammatory bowel diseases are a group of disorders that are polygenic in origin and involve intestinal epithelial breakdown. We investigated the possibility that these keratin mutations might contribute to the course of the disease by adversely affecting the keratin filament network that provides mechanical support to cells in epithelia. The mutations (Gly62 to Cys, Ile63 to Val and Lys464 to Asn) all lie outside the major mutation hotspots associated with severe disease in epidermal keratins, but using a combination of in vitro and cell culture assays we show that they all have detrimental effects on K8/K18 filament assembly in vitro and in cultured cells. The G62C mutation also gives rise to homodimer formation on oxidative stress to cultured intestinal epithelial cells, and homodimers are known to be polymerization incompetent. Impaired keratin assembly resulting from the K8 mutations found in some inflammatory bowel disease patients would be predicted to affect the maintenance and re-establishment of mechanical resilience in vivo, as required during keratin cytoskeleton remodeling in cell division and differentiation, which may lead to epithelial fragility in the gut. Simple epithelial keratins may thus be considered as candidates for genes contributing to a risk of inflammatory bowel disease.

Key words: Inflammatory bowel disease, K8 mutations, Chromosome 12q, IBD2, Crohn disease, Ulcerative colitis

Introduction

The keratins are a family of structural proteins that form the intermediate filaments of the cytoskeleton in epithelial cells. At least 49 keratin genes are present in humans (Hesse et al., 2001) and these genes are expressed in (type I + type II) pairs, as the proteins form obligate heteropolymeric filaments. Each keratin pair has a specific and characteristic tissue distribution pattern. Although the thick external barrier epithelia express K1-K6 (type II keratins) and K9-K17 (type I), internal epithelia like that of the intestine express principally K8 (type II) and K18 (type I), sometimes with K7, K19 or K20. Most type I keratin genes are on chromosome 17q, but the type I gene for K18 (*KRT18*) is located on chromosome 12q (Waseem et al., 1990), near the type II genes and adjacent to the *KRT8* gene.

Human hereditary skin disorders caused by mutations in keratin genes have shown that keratin intermediate filaments in stratified squamous epithelia provide vital mechanical resilience to the keratinocytes, without which the epithelium breaks down under physical stress (reviewed by Corden and McLean, 1996). Mutations in keratins K5 (type II) or K14 (type I), synthesized in the basal cell layer of the epidermis, cause epidermolysis bullosa simplex (EBS), a condition characterized by basal cell cytolysis and epidermal blistering in response to mild physical trauma. Cell fragility varies according to the position of the mutation in the keratin protein, with mutations in the 'hotspots' at either end of the central rod domain (the building block of the filament) producing the most severe disease (Letai et al., 1993). Similar mutations in other differentiation-specific keratins result in a range of very different clinical phenotypes, depending on which subpopulation of cells in the tissue uses the mutated keratin for its structural resilience (reviewed by Irvine and McLean, 1999).

Compared with the epidermal keratins, there is less evidence to date for human pathogenic mutations in K8 or K18, the major keratins of simple epithelia. Some human mutations in K8/K18 have been associated with cryptogenic cirrhosis and other liver disorders (Ku et al., 2003; Ku et al., 2001; Ku et al.,

1997) and recently with pancreatitis (Cavestro et al., 2003), suggesting that K8/K18 may also be involved in human pathology outside the liver. The absence of functional K8 or K18 predisposes mice to hepatotoxic damage (Magin et al., 1998; Zatloukal et al., 2000), but depending on genetic background, the lack of K8 in mice can also cause either embryonic lethality or colonic hyperplasia and anorectal prolapse (Baribault et al., 1994).

Mutations in human K8 and K18 genes may contribute to disorders of internal systems without this being readily apparent, because early lesions caused by cell fragility in internal simple epithelia would not be visible. Intestinal epithelium is a probable 'candidate tissue' for K8/K18 pathology for two reasons: first, because diseases in external epithelia have clearly shown that keratin mutations lead to cell lysis on physical stress, and among internal epithelia, the intestine is probably subjected to the greatest physical stress, and second, as discussed above, there is an already reported intestinal phenotype in the K8 knockout mice. We reasoned that even if cell fragility was involved in gut disorders, the lack of access and visibility of the gut epithelium would mean that primary pathology might go undetected. An internal epithelial fragility disorder might not present clinically until secondary effects had become established (such as a dysregulated immune response to entry of gut lumen antigens into the intestinal wall), and the tissue damage had become so extensive as to obscure the primary cause.

A candidate group of gut disorders for simple epithelial keratin involvement is that of the inflammatory bowel diseases (IBD). IBD is a heterogeneous group of disorders of poorly understood aetiology affecting 400 individuals per 100,000 in the United Kingdom (Rubin et al., 2000). It is characterized by an ill-defined increase in intestinal permeability and an abnormal inflammatory response, with a polygenic determination of the disease type and severity. The two main forms are Crohn disease (Crohn's disease, OMIM 266600) and ulcerative colitis, and the quest to identify the genes responsible for these disorders has been ongoing for some time. Genetic linkage with IBD has been reported for loci on chromosomes 3, 5, 6, 7, 12, 14, 16 and 19 (Hugot et al., 1996; Hugot et al., 1999; Rioux et al., 2000; van Heel et al., 2000). The strongest genetic linkage with IBD is between the *IBD1* locus (OMIM 266600) on chromosome 16 and Crohn disease. One of the genes contributing to this linkage has now been identified as *CARD15* (*NOD2*) (Hugot et al., 2001; Lesage et al., 2002; Ogura et al., 2001). This gene encodes a receptor for a common peptidoglycan motif on all bacteria, the muramyl dipeptide (Girardin et al., 2003), and sequence variants associated with Crohn disease are defective in this recognition.

The second most strongly linked locus, *IBD2* (OMIM 601458) of chromosome 12q (Parkes et al., 2000; Satsangi et al., 1996), includes the cluster of the type II keratin genes of the intermediate filament gene superfamily. Some candidate genes in this IBD2 locus have now been excluded, such as those encoding β7 integrin (van Heel et al., 2001), natural resistance associated macrophage protein 2 (Stokkers et al., 2000), STAT6 (signal transducer and activator of transcription 6) (Xia et al., 2002) and advillin (Tumer et al., 2002), but no keratins so far.

The linkage of inflammatory bowel disorders to multiple genetic loci indicates that there are likely to be many susceptibility genes involved, either contributing to molecularly distinct subsets of IBD that cannot be separately identified clinically, or perhaps contributing in different combinations to cause the same disease. Any single genetic change is therefore unlikely to be present at a very high frequency in patients with inflammatory bowel disorder and may also be detected in healthy controls. These considerations make the problem of identification and analysis of causative mutations especially difficult.

We sequenced the DNA of *KRT8* and *KRT18* from several familial and sporadic IBD patients with either ulcerative colitis or Crohn disease. We found sequence variations in the K8 gene in both ulcerative colitis and Crohn disease patients, and we present experimental evidence that these mutations have significant effects on keratin filament assembly. The data suggest that these mutations have the potential to compromise cytoskeleton function in the intestinal epithelium in vivo. By analogy with the effects of mutations in skin keratins, the K8 mutations may render intestinal epithelia fragile, providing a mechanism whereby keratins may be one of the genetic factors which, in some patients, predispose them towards inflammatory bowel disorders.

Materials and Methods

DNA sequencing

Genomic DNA from ulcerative colitis or Crohn disease patients was collected in the Wellcome Trust Centre for Human Genetics, Oxford or prepared from blood samples collected at the Gastrointestinal Laboratory, Western General Hospital, Edinburgh. Exonic sequences of K8 and K18 were PCR amplified using total genomic DNA template with primers homologous to flanking intronic sequences. PCR products were purified using Boehringer HighPure PCR purification kit (Roche Diagnostics, Lewes, UK), sequenced using the ABI Rhodamine Terminator Cycle Sequencing Ready Reaction kit (PE Biosystems, Warrington, UK) and analysed on an ABI 377 sequencer.

K8 and K18 proteins

K8 cDNA clones were generated from MCF-7 (human mammary carcinoma cell line) mRNA by reverse transcription, PCR amplified, ligated into pGEM-T Easy (Promega, Southampton, UK) and sequenced. Human K18 cDNA was a gift from M. Bishr Omary (Stanford University). Site-directed mutagenesis was performed using the QuikChange kit (Stratagene, Cambridge, UK) to generate K8/K18 with patient mutations. Human K8 (wild type), K8(G62C), K8(I63V), K8(K464N), K18 (wild type) and K18(S230T) cDNAs were generated with an *Nde*I restriction site immediately before the start codon using a PCR 5′-primer with a 3 basepair extension of CAT. After sequencing, cDNAs were cloned into pET-23a(+) vector (Cambridge Bioscience, Cambridge, UK) and individually transformed into *Escherichia coli* strain BL21(DE3) pLysS. Keratins were expressed and purified as described previously (Porter et al., 1998).

In vitro assembly assays

Filament assembly was initiated by dialysing 75 μg/ml of K8 and K18 protein at a 1:1 weight ratio against 10 mM Tris-HCl, pH 7.3, 2 mM ethylenediaminetetraacetic acid (EDTA), 25 mM 2-mercaptoethanol for 30 hours. Efficiency of assembly was assessed by sedimentation assay (Nicholl and Quinlan, 1994). The assembly mixture was layered onto 0.85 M sucrose in assembly buffer and centrifuged for 30 minutes at 80,000 ***g*** at 20°C in a TLS-55 rotor using a Beckman

TL100 ultracentrifuge. Proteins were precipitated from supernatants; pellets were dissolved in sample buffer volumes proportional to the original sample volume for SDS-PAGE comparison using 7% NuPage pre-cast Tris-Acetate resolving gels with SeeBlue Plus2 prestained markers (Invitrogen, Paisley, UK). For electron microscopy, protein samples were diluted to 15 µg/ml and negatively stained with 1% (w/v) uranyl acetate.

Affinity measurements

Affinities of wild-type and mutant K8/K18 combinations were determined using surface plasmon resonance analysis with a Biacore 3000 apparatus (Biacore AB, Uppsala, Sweden). Purified K8 and K18 proteins were dialysed stepwise against 4 M urea, 10 mM Tris-HCl, pH 7.3, 2 mM EDTA, 1 mM dithiothreitol (DTT) then 2 M urea, 1 M urea, 0.5 M urea then 10 mM Tris-HCl, pH 7.3, 2 mM EDTA, 1 mM DTT over 48 hours. Protein concentrations were determined by Bradford protein assay (Bio-Rad, Hemel Hempstead, UK) before dilution. K8 (wild type) or K18 (wild type) were covalently immobilized to a CM5 sensor chip in HBS-EP (10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (HEPES), pH 7.4, 0.15 M NaCl, 3.4 mM EDTA, 0.005% surfactant P20, 1 mM DTT) at a flow rate of 5 µl/minute. K8 or K18 was injected at 50 µg/ml in 10 mM acetate, pH 4.5 and unreacted groups were then blocked by injection of 1 M ethanolamine, pH 8.5. Proteins for binding to immobilized keratins were diluted to 20 µg/ml (in HBS-EP, 1 mM DTT), injected at a flow rate of 5 µl/minute for 5 minutes at 25°C then washed for 5 minutes. Binding of injected keratin to immobilized keratin was compared with a parallel 'naked' chip to measure nonspecific binding to the matrix. The sensor chip was regenerated between injections by washing with 5 µl of 6 M guanidine hydrochloride in HBS-EP buffer.

K8 and K18 eukaryotic expression and oxidative stress

Wild-type or mutant K8 and K18 cDNAs were cloned with or without a FLAG epitope tag into pcDNA3 vector (Invitrogen). 1.5 µg of untagged wild-type K8 or mutant K8 DNA was transiently cotransfected with 1.5 µg of untagged wild-type K18 per well (6-well plates) of SW13 (adrenal cortex adenocarcinoma) cells using FuGene (Roche). For immunofluorescence, SW13 cells were fixed in –20°C methanol for 5 minutes and stained with anti-keratin 8 monoclonal antibody M20 (ICN, Basingstoke, UK) followed by Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes). SW480 cells (human colorectal adenocarcinoma) stably expressing FLAG-tagged K8(G62C) were derived from single cell clones after selection of transfected cells with 0.75 mg/ml geneticin (Invitrogen). For oxidative stress experiments, SW480 cells were treated with 20 mM H_2O_2 for 1 hour, washed twice with phosphate buffered saline, lysed in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, separated on nonreducing or reducing 10% SDS-PAGE with Magic Markers (Invitrogen) and immunoblotted using anti-FLAG monoclonal antibody M2 (Sigma, Poole, UK) or anti-K8 antibody M20. Bound antibody was detected by enhanced chemiluminescence.

Results

K8 and K18 sequence variants in ulcerative colitis and Crohn disease

The complete coding regions of the keratin 8 (K8) and keratin 18 (K18) genes, *KRT8* and *KRT18*, respectively, were sequenced from genomic DNA samples collected from 50 sporadic and 47 familial inflammatory bowel disorder patients. A total of 57 patients were diagnosed as having Crohn disease, 32 as having ulcerative colitis and 8 as having indeterminate colitis. Heterozygous miss-sense mutations in keratin K8 were found in five unrelated inflammatory bowel disease patients (5.2%) identified by comparison with published K8 (Waseem et al., 1990) and K18 (Kulesh and Oshima, 1988) sequences.

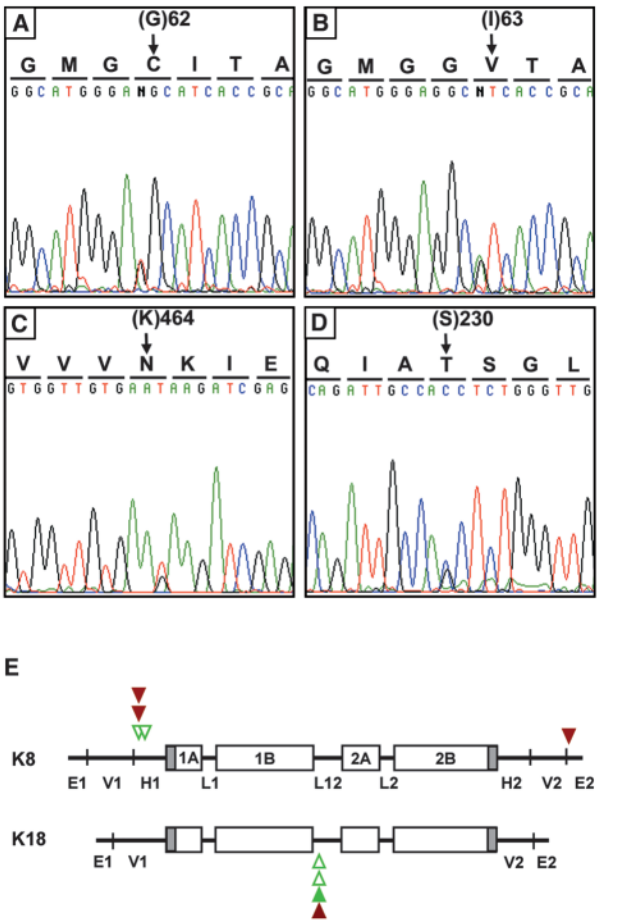

Fig. 1. Detection of K8 and K18 sequence variations in inflammatory bowel disease patients. (A) Patient DNA sequence from an ulcerative colitis patient showing heterozygous KRT8 g.1296G>T causing K8 Gly-62→Cys. (B) Patient DNA sequence showing heterozygous KRT8 g.1299A>G causing K8 Ile63→Val. (C) Patient DNA sequence showing KRT8 g.8510G>T causing K8 Lys464→Asn. (D) Patient DNA sequence showing KRT18 g.4918G>C causing K18 Ser230→Thr. (E) Positions of the amino acid substitutions in the K8 and K18 proteins. The helical domains are represented by boxes (shaded regions are hotspots for mutations causing severe disease in epidermal keratins) and the non-helical domains are represented by the solid line. Solid red triangles represent familial ulcerative colitis, solid green triangles represent familial Crohn disease and open green triangles represent sporadic Crohn disease.

Three separate incidences of the *KRT8* mutation leading to amino acid substitutions at K8(G62C) were identified (counting the initiating methionine as +1) (Dunnen and Antonarakis, 2000); two were in unrelated ulcerative colitis patients with a family history of IBD and one was in a Crohn disease patient with no family history (Fig. 1A). In one familial incidence, the K8(G62C) proband's UC-affected sister also expressed the mutation, as did the brother who had symptoms of colitis but had not been formally diagnosed. This mutation

lies in the non-helical head domain of the keratin protein, in subdomain H1, and is homologous to the K8(Gly61 to Cys) mutation reported in liver disease patients (Ku et al., 2001). A mutation in the adjacent codon K8(Ile63 to Val) was found in a sporadic Crohn disease patient (Fig. 1B). A third mutation leading to an amino acid substitution at K8(Lys464 to Asn) in the tail domain was found in an ulcerative colitis patient with a family history of IBD (Fig. 1C). One heterozygous *KRT18* sequence variant of potential interest was identified in four patients (Fig. 1D), leading to K18(Ser230 to Thr) in the L12 linker region. The positions of the sequence variants in the protein are shown in Fig. 1E. None of them are in the major hotspot cluster sites associated with severe disease in epidermal keratins. Instead, they lie within the non-helical domains, which in the skin keratins are usually associated with milder forms of disease (Letai et al., 1993).

With the known polygenic etiology of IBD, a population incidence of 1 in 250 and a typical pattern of onset in young adulthood, one would predict that predisposing but asymptomatic genetic changes should be found in some unaffected individuals, particularly in young ones. When unaffected control samples were screened by direct sequencing for the presence of keratin mutations, the K8(K464N) mutation was not found in 194 control alleles, but the K8(G62C) and K8(I63V) sequences were each detected once, the latter in a young individual. These individuals may be presymptomatic for IBD, or for cirrhosis of the liver (Ku et al., 2001; Ku et al., 1997), or they may possess only one of the multiple susceptibility genes required to succumb to the disorder. The K18(S230T) variant was detected altogether in 4 out of 194 alleles and may therefore be a polymorphism rather than a mutation. With this degree of complexity in the system, statistical analysis on this sample size is not informative. Tissue samples could not be obtained from these patients, and in any case they may not have shown much morphological change in the cytoplasmic keratin filaments. These mutations occurred outside the helix boundary motifs, and from studies of mutations in skin keratins, it is only the rod end hotspot mutations that have been associated with any obvious keratin dysmorphism in situ. We therefore focused our analysis of the potential pathogenicity of these mutations on their impact on the keratin cytoskeleton in vitro.

Mutant keratins show inefficient filament assembly in vitro

As all the mutations detected affect amino acids outside the major disease hotspot sites identified in epidermal keratins, it was necessary to see whether the K8/K18 mutations were likely to impair filament function. We initially assessed the assembly potential of IBD mutant versus wild-type recombinant K8 with wild-type recombinant K18 using sedimentation assays and electron microscopy of negatively stained filament preparations as previously used to show deficiencies in keratins with EBS mutations (Coulombe et al., 1991).

Recombinant proteins produced in bacteria were purified (Fig. 2A) and dialysed together in equimolar amounts of K18 and K8. Under reducing conditions, most of the wild-type (K8 + K18) protein was recovered in the pellet fraction of the sedimentation assay, indicating that the protein had polymerized successfully (Fig. 2B). However, none of the K8 mutants assembled as efficiently with wild-type K18 as did wild-type K8; much of the protein remained in the supernatant and a smaller proportion was recovered in the pellet. In this assay, K18(S230T) was indistinguishable from wild-type K18 (Fig. 2C).

By electron microscopy, wild-type K18 + K8 formed abundant long, smooth, regular filaments of constant diameter, showing frequent lateral associations between filaments (Fig. 2D); filament ends and unpolymerized material were hardly ever seen. By contrast, polymerization with mutant K8 produced shorter filaments of less uniform diameter; these filaments were less straight, made sharper bends and showed fewer lateral associations than the wild-type filaments (Fig. 2E-G). More aggregates and short fragments were invariably present, indicating that the polymerization efficiency of these proteins is impaired. In combination with wild-type K8, the K18(S230T) variant produced filaments more similar to wild-type K18-based filaments (Fig. 2H), again consistent with the interpretation that this is a polymorphism rather than a pathogenic mutation.

IBD mutations alter heterodimer formation

Because the in vitro analyses described above do not discriminate between the different stages of keratin filament assembly, we sought to determine the effects of the sequence variants on the first stage of assembly, i.e. the formation of the type I/type II keratin heterodimer. Surface plasmon resonance analysis was undertaken with recombinant wild-type K8 or K18 covalently bound to the sensor chip and exposed to recombinant K18 or K8 solutions, respectively, flowing across the chip under reducing conditions. The optical detection system read-out depends on the mass of protein bound to the chip (Fig. 3). At 5 μl/min exposure over 5 minutes, binding to the immobilized keratin was not saturated. No binding of a nonkeratin control protein (BSA) to the immobilized K8 or K18 was detected. No dissociation of any of the keratin-keratin interactions was observed within the 5 minute wash period, reflecting the high affinity of type I-type II keratin interactions.

By 5 minutes the immobilized K18 had the highest bound levels of wild-type K8 and the lowest of K8(G62C) (70% of wild-type) followed by K8(K464N) (a mutation which is closer to the C-terminus than any previously reported keratin mutation), as shown by the slope of the curve (Fig. 3A). The other two sequence variants revealed differences between defects seen in dimerization rates (this assay) and in their ultimate efficiency of filament formation (see above). Binding of K8(I63V) to K18 was not significantly different from wild-type K8 under these conditions, although in vitro assembly over a longer timecourse had shown abnormalities by electron microscopy. K18(S230T) binding to K8 was about 72% that of wild-type K18 at 5 minutes (Fig. 3B), although filament formation looks normal at longer timepoints. The different behaviour of individual sequence variants in the different assays probably reflects the different stage in hierarchical protein folding and filament assembly at which the conformational effect of the mutation has most impact.

These experiments indicate that mutant keratins K8(G62C), K8(K464N), and to a lesser extent K18(S230T), are inherently abnormal in the process of assembling heterodimers in this

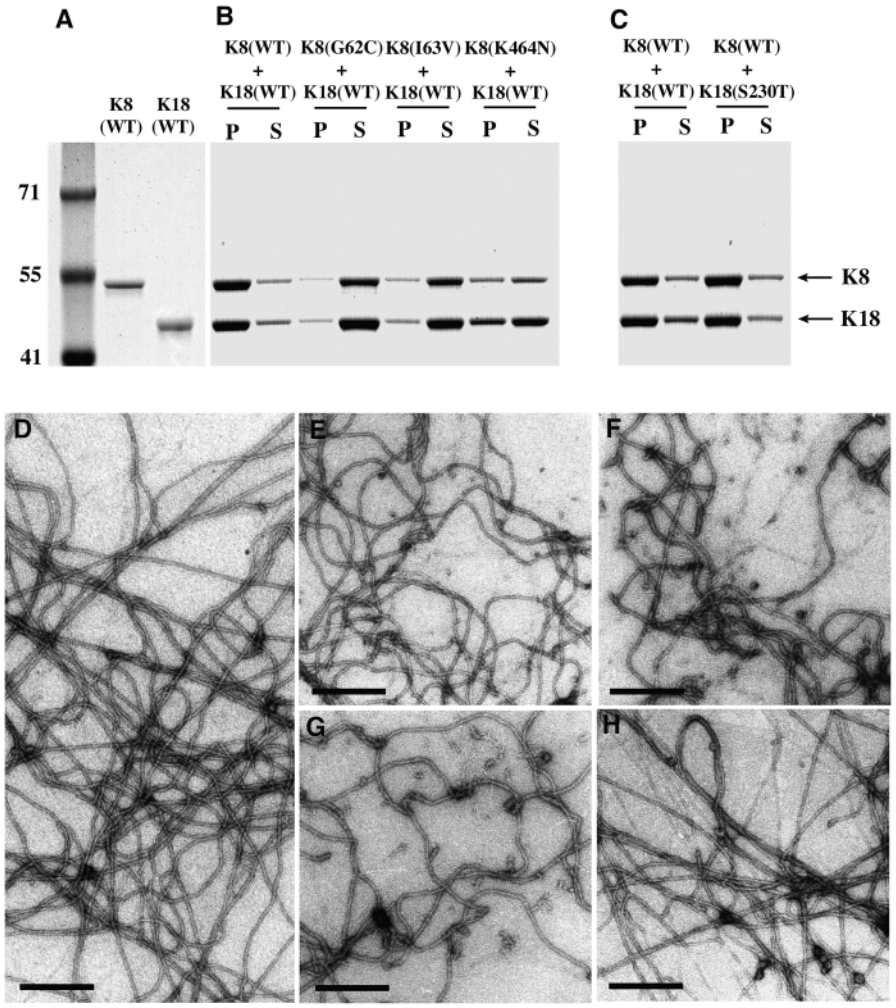

Fig. 2. In vitro filament assembly properties of variant keratins. (A) SDS polyacrylamide gel electrophoresis plus Coomassie Blue staining to show (left to right) molecular weight markers (Mr in kDa as indicated), recombinant K8(wt) and K18(wt), respectively. (B) Coomassie Blue-stained SDS-PAGE of samples from a sedimentation assay of K8(wt), K8(G62C), K8(I63V) or K8(K464N) each with K18(wt), polymerized in vitro under reducing conditions. 'P' denotes pelleted (i.e. filamentous) material; 'S' denotes material recovered from the supernatant. K8(wt) and K18(wt) readily assemble into pelletable polymers, whereas the mutant K8 forms smaller amounts of pelleted material in the sedimentation assay. (C) Coomassie Blue-stained SDS-PAGE of a sedimentation assay of K8(wt) with K18(wt) or K18(S230T), polymerized in vitro under reducing conditions: K18(S230T) mutant behaves as wild type. (D) Electron micrograph showing long, uniform filaments formed by in vitro polymerization of K8(wt) with K18(wt). Polymerization of wild-type K18 with (E): K8(G62C), (F): K8(I63V) or (G): K8(K464N) produces shorter, less uniform filaments and more small particulate aggregates. (H) K18(S230T) forms filaments with K8 (wild type) that are similar to those formed by wild-type K18. Bars, 200 nm.

assay system. Yet although the mutations affect the rate at which heterodimers initially form, they do not weaken the affinity between subunits once they are assembled into heterodimers as there is no dissociation of any of them during the wash period.

K8(G62C) polymerization de novo is impaired

We carried out transient transfection studies to determine whether the keratin sequence variants also perturbed filament assembly and organization in the cellular environment. Intermediate filament proteins with substantial deletions and disruptive mutations can still become incorporated into a pre-existing cytoplasmic filament network (for example, see Lu et al., 1993), so we used the more stringent test of assembling a filament network de novo in cells with no pre-existing filaments. SW13 adrenal cortex adenocarcinoma cells are reported to contain no cytoplasmic intermediate filaments (Hedberg and Chen, 1986) and so were used for these experiments.

SW13 cells were cotransfected with constructs expressing wild-type or variant K8, together with wild-type or variant K18, then fixed at intervals after transfection and the keratin visualized by immunofluorescence (Fig. 4). In cells transfected with K8(G62C)+K18, the keratin was mostly in the form of aggregates at 24 hours after transfection (Fig. 4B), but the wild-type keratins (Fig. 4A) were mainly filamentous. The other keratin variants were less obviously different from wild type (not shown). By late timepoints (120 hours) the wild-type keratins were unchanged, while the keratin involving K8(G62C) was nearly all in the form of filamentous fragments with infrequent aggregates (Fig. 4C,D). Cells expressing the K8(I63V), K8(K464N) and K18(S230T) were not significantly different from wild-type cells at either timepoint. This experiment supports the interpretation that K8(G62C) is clearly a disruptive variant, but that filament assembly is still only impaired by this mutation rather than prevented.

Oxidative stress increases K8(G62C) homodimer formation in cultured colon cells

Keratin filaments normally only form as heterodimeric polymers, with a type I and a type II subunit forming a coiled-coil heterodimer along their rod domains as the first stage of assembly. However, K8 and K18 have both been shown to form transient low-affinity homodimers in pure single protein solutions (Quinlan et al., 1986). Neither K8 nor K18 has any cysteine residues in the wild-type sequence, but experimental introduction of a cysteine residue into the rod domain of K8 resulted in the formation of stable disulphide-linked

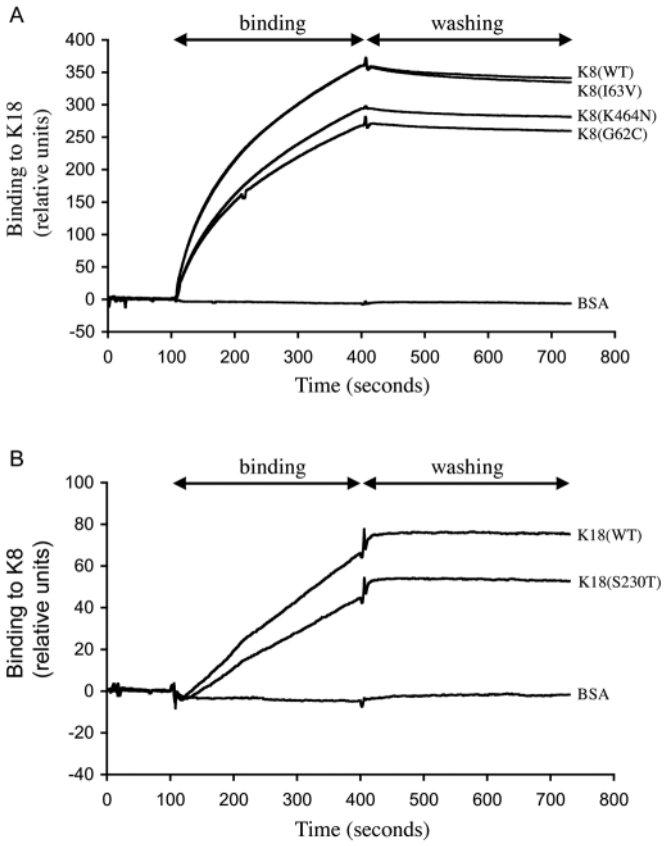

Fig. 3. Plasmon resonance analysis of K8/K18 binding. (A) Binding of 20 μg/ml K8 (wild type), K8(G62C), K8(I63V), K8(K464N) or BSA to immobilized wild-type recombinant K18 protein for 5 minutes, followed by washing for 5 minutes, under reducing conditions. K8(G62C) and K8(K464N) binding was less efficient than K8 (wild type), and K8(I63V) was similar to wild type. (B) Binding of K18(S230T) to wild-type immobilized K8 was impaired compared with K18 (wild type), and BSA binding was negligible. Once formed, all K8-K18 interactions were stable as shown by the persistent plateau through the wash period.

homodimers, which were unable to participate in filament assembly (Hatzfeld and Weber, 1990). The cysteine residue in K8(G62C) is not within the rod domain and it is not known whether the head domains would ever get close enough for disulphide bonds to form between such cysteines. However, if stable homodimers were to form in vivo, this might increase the detrimental impact of this mutation further.

To test whether K8(G62C) can make homodimers in an appropriate cellular environment, we used SW480 human colorectal adenocarcinoma cells stably transfected with FLAG-tagged K8(G62C). Transfected cells were exposed to 20 mM H_2O_2 for 60 minutes before harvesting total cell protein for analysis by immunoblotting. This short oxidative pulse has been shown to increase the rate of disulphide bond formation between cellular proteins (Zavialov et al., 1998). In transfected cultures, the anti-FLAG antibody M2 detected a protein with an apparent M_r slightly larger than endogenous K8 (54 vs 52 kDa) and which was absent from nontransfected cells (Fig. 5). In cells expressing K8(G62C), an M2-positive band with an apparent molecular weight of just over 100 kDa was also visible in cells subjected to oxidative stress (Fig. 5A). This band was only present in extracts separated under nonreducing conditions (compare Fig. 5A with 5B), supporting the interpretation that it represents disulphide-linked K8(G62C) homodimers. The endogenous K8 present in nontransfected SW480 cells did not form putative homodimers (Fig. 5C).

Thus, K8(G62C) homodimerization can take place in living cells in conditions of oxidative stress. As keratin homodimers are not competent to form filaments, homodimer formation may reduce the amount of filamentous keratin in the cell and should be considered as a potential contributing factor to the pathogenic properties of this particular mutation.

Discussion

In this paper we report the finding of mutations in K8, one of the two primary keratins of simple epithelia, in a subset of patients with inflammatory bowel disease. We show that these mutations can alter the dynamics of assembly of the keratin cytoskeleton in a manner that is predicted to be detrimental to the resilience of intestinal epithelial cells in situ. We suggest that these observations highlight another group of candidate susceptibility genes, i.e. the intestinal simple epithelial keratin genes, for consideration and further analysis in a multi-hit polygenic model of inflammatory bowel disease.

The scope for tissue analysis of keratin mutations

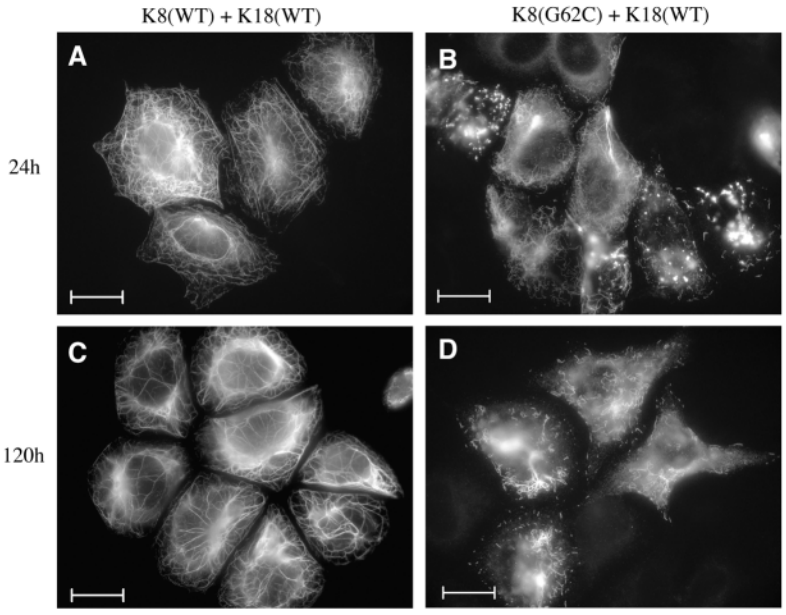

Fig. 4. De novo filament assembly in transfected keratin-free SW13 epithelial cells. At 24 hours after transfection, most cells coexpressing K8 (wild type) and K18 (wild type) contained extended filament networks (A), whereas most cells coexpressing K8(G62C) and K18 (wild type) contained short lengths of disorganized filaments and aggregates (B). By 120 hours after tranfection with wild-type K8 and K18, most transfected cells contained extended arrays of ordered keratin filaments (C). However, cells expressing the K8(G62C) mutant contained predominantly disordered filament fragments (D). Bar, 20 μm.

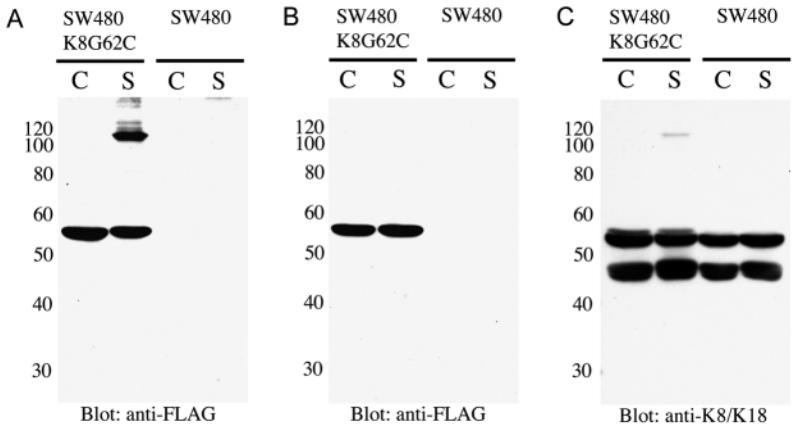

Fig. 5. Oxidative stress of nontransfected SW480 intestinal epithelial cells and SW480 cells stably expressing FLAG-tagged K8(G62C). Extracts were prepared from control cells incubated under normal growing conditions 'C' or from cells subjected to oxidative stress 'S' by treatment with 20 mM H_2O_2 for 1 hour. (A) Anti-FLAG immunoblot of proteins extracted from cells separated under nonreducing conditions. FLAG-tagged K8(G62C) was only detected in the transfected cultures and a putative K8 homodimer with an apparent molecular weight of just over 100 kDa was also detected in cells expressing K8(G62C) after oxidative stress. (B) Same samples as in (A) but run under reducing conditions: the high Mr band in K8(G62C) cells is no longer apparent as the disulphide bond in the homodimer has been reduced and all the K8 runs as monomers. (C) Immunoblotting extracts run under nonreducing conditions with monoclonal antibody M20 (detects K8 and K18) showed that endogenous K8 did not form putative homodimers in nontransfected cells. The monomeric FLAG-tagged K8(G62C) is detected by M20 as a weaker signal than endogenous K8 with an apparent molecular weight of 54 kDa in the unstressed transfected cells. The homodimeric FLAG-tagged K8(G62C) is also detected by M20 in the stressed cells.

is much more limited for simple epithelial keratins than for epidermal keratins. Unlike the situation in EBS and other skin blistering disorders, samples of affected or at-risk internal epithelia are not often available. Early lesions or small areas of epithelial breakdown are not readily visible as they are in skin. At the time of first clinical presentation by IBD patients, intestinal tissue damage is already extensive. If biopsy material were to become available from a patient with one of these keratin mutations, the chances of seeing any abnormality in the filaments would be small. Only the most severe mutations produce clearly abnormal keratin structures in the epidermal disorders, even at the electron microscopic level. Finally, inflammatory bowel disorders are known to be polygenic in origin, and so will never show the clear-cut relationship between mutation and phenotype that has been seen in the skin fragility disorders. Until we know more about the other genes involved, the development of transgenic animal models with keratin mutations is also unlikely to be a productive approach.

For these reasons we focused our functional analysis on the properties of the mutant keratin proteins in vitro and in cultured cells, to determine whether they show properties that might be expected to weaken the keratin cytoskeleton of any cell expressing them. We chose a series of assays that measure different aspects of keratin behaviour: the sedimentation assay measures the extent, and the EM assay the quality, of polymerization over a long timespan, whereas the surface plasmon resonance measurements reflect the real-time rate of dimer formation, the essential first step in assembly. Filament formation within transfected SW13 cells addresses the efficiency of de novo filament assembly in the cellular environment. Different mutations will affect these parameters in different ways as the protein domains contribute to different stages of assembly. These assays have shown that all the K8 sequence variants identified are still capable of making heteropolymeric filaments, although they do so less efficiently than wild-type K8, and that the K8(G62C) mutation is undoubtedly the most defective of this group.

K8 mutations in IBD patients are predicted to be 'mild'

There is now a clear and well-established association between keratin mutations and epithelial tissue fragility disorders, implicating at least 14 different keratin genes in the etiology of 18 or more clinically distinct disorders caused by defects in single genes (The Human Intermediate Filament Mutation Database, http://www.interfil.org/). The keratin mutations are unequally distributed along the protein sequences and the severity of the clinical phenotype arising from these mutations depends to an extent on the position of the mutation in the keratin protein. Important 'hotspots' at the ends of the rod domain (Fig. 1E) account for over 75% of pathogenic keratin mutations to date (Porter and Lane, 2003) and cause the most severe disease phenotypes associated with mutations in the primary keratinocyte keratins K5 and K14. We did not find this type of mutation in K8. In the epidermis, mutations outside these rod ends, either elsewhere in the rod domain or in the non-helical head, tail and linker domains, are associated with milder phenotypes. The K8 mutations described here occur in the head and tail domains and this, together with the experimental data, suggests that these sequence variants are most likely to cause a mild phenotype of cytoskeleton dysfunction.

It is possible that only mild mutations in K8 are viable in humans. K8 is expressed in early embryos and is a major constituent of the placental intermediate filaments. Mice with no K8 show very low viability in utero (depending on genetic background) (Baribault et al., 1994) and total loss of function of K8 may be lethal in humans. Severe K8 rod end mutations would therefore probably also be embryonic-lethal in humans, and thus never detected in live births. 'Mild' K8 mutations may be more easily tolerated but mild mutations may be asymptomatic in the absence of compounding factors, genetic or environmental, which would render them harmful at later stages of development.

Our results suggest that there are two ways in which filament assembly in intestinal epithelium could be perturbed by K8 head and tail domain mutations: by altering K8 conformation to interfere with the efficiency of filament formation, and in the case of the more common K8(G62C), additionally by homodimer formation, which may lead to depletion of the assembly competent pool of subunits in the cell, particularly during oxidative stress.

Possible consequences of altered K8 conformation

The experiments presented here show that single amino acid changes in the K8 protein sequence, particularly K8(G62C), can alter the kinetics of keratin dimer formation and filament network formation. The position of the H1 domain mutations G62C and the less detrimental I63V are similar, although not identical, to some mutations previously reported in skin keratins. Mutations in conserved residues of the H1 domain in K1 have been identified in patients with bullous congenital ichthyosiform erythroderma (epidermolytic hyperkeratosis) (Chipev et al., 1992; Whittock et al., 2001), and there are several examples of mutations in the H1 domain of K5 that are associated with mild forms of epidermolysis bullosa simplex (Muller et al., 1998; Rugg et al., 1995).

The Gly62 and Ile63 residues of K8 are conserved throughout the type II keratins (Fig. 6), except for the divergent hair keratins, and lie at the transition between the V1 (variable) and the H1 (hypervariable) subdomains in the non-helical head domain of the protein. Head domains of intermediate filaments are known to be essential for filament assembly (Hatzfeld and Burba, 1994; Herrmann et al., 1992) and are now being increasingly implicated in interactions between intermediate filaments and a variety of other proteins (Candi et al., 1998; Meng et al., 1997). The H1 domain, and H2 in the tail, flank the α-helical rod domain and contain phosphorylation sites essential for remodelling the filament networks. An increase in the phosphorylation state favours disassembly (Ando et al., 1989). Thus, the tertiary conformation of this H1 domain is crucial for efficient filament formation. For example, Ser74 is a key residue that is phosphorylated in mitosis and in cells under stress (Ku and Omary, 1997), and the Gly62 and Ile63 are only 11-12 residues away. The G62C and I63V mutations may alter this region to either mimic or inhibit phosphorylation-dependent conformational changes needed for assembly/reassembly during the cell cycle.

The Lys464 of K8 lies at the other end of the protein and is closer to the carboxy-terminus than other keratin mutation sites; like Gly62 and Ile63, it is conserved among type II keratins. The K464N mutation has a negative effect on both in vitro assembly and on the rate of dimer formation. Thus, the surface plasmon resonance analysis shows that correct sequence features in the head and tail domains are crucial for the efficient interaction of type I with type II monomers to form dimers.

Unlike the K8 residues described above, K18 Ser230 is not conserved among type I keratins, suggesting that this is not a critical residue. This is consistent with our data showing that the S230T substitution has little effect on eventual keratin filament assembly, although it appears to retard dimer association in the surface plasmon resonance analysis.

Homodimer formation may reduce ‘usable’ keratin

We have shown that the K8(G62C) mutant protein is able to form homodimers that are stabilized by intermolecular disulphide bonds, and that this happens not only in an in vitro assembly mixture but also in the cytoplasm of living intestinal epithelial cells that have been transfected with the mutant keratin sequence. This may shed further light on the conformation of the H1 domain, as it implies a very close proximity between the two H1 domains in a homodimer.

Human K8	GGYGGASGMG**GI**TAVTVNQSLL
Mouse K8	SGGFGGAGVG**GI**TAVTVNQSLL
Xenopus K8	GFGGAGVGSA**GI**TSVSVNQSLL
Human K1	GGYGPVCPPG**GI**QEVTINQSLL
Human K5	GPGFPVCPPG**GI**QEVTVNQSLL

Fig. 6. Protein sequence alignment of type II keratins in the head domain V1/H1 region. Gly62 and Ile63 (bold type) are conserved in all type II keratins, as are the other shaded amino acids, suggesting that substitutions at these locations are likely to have functional consequences.

Homodimer formation is likely to be inhibitory to filament assembly as homodimers do not become incorporated into a filament structure (Hatzfeld and Weber, 1990). We have also shown that homodimer formation increases under conditions of oxidative stress, which may exist at sites of inflammation in vivo. The effect of the K8(G62C) mutation is therefore likely to be exacerbated in people with inflammatory bowel disorders, in whom oxidant-induced injury to the intestinal epithelial cells is known to occur (Keshavarzian et al., 2003; McKenzie et al., 1996). This K8(G62C) mutation was the most frequently observed sequence variant and it is interesting to note that this is the most common mutation that Omary and colleagues have identified in liver disease patients (Ku et al., 2003; Ku et al., 2001). This supports the likelihood that this mutation is indeed significantly pathogenic.

Consequences of impaired assembly

The pathogenic mutations identified in epidermal keratins are clearly associated with cell fragility and tissue breakdown. The primary effect of the mutations identified here in some IBD patients would probably be to reduce the efficiency with which the filaments can form in the first place, although dynamic subunit exchange on filaments also takes place in living cells (Miller et al., 1991) and could also be affected by these mutations. A feature of the intestinal epithelium is its high proliferative capacity and rate of turnover. During cell division, intermediate filament proteins become destabilized by transient hyperphosphorylation to allow for remodelling. This is important for many aspects of mitosis, from nuclear envelope breakdown to repositioning of daughter cells without the normal constraints of keratin-desmosome connections. During mitosis the epithelial cytoskeleton is thus temporarily ineffective, creating a window of cell vulnerability. If the assembly of proteins into new filaments after cell division is retarded by mutations, then this window of fragility could be greatly extended, leaving the tissue especially fragile.

There is also growing evidence of a role for keratin in cell signalling, particularly with modulation of tumour necrosis factor-α (TNFα) and Fas-driven apoptosis. Keratins 8/18 interact with Fas and TNFα receptors (Caulin et al., 2000; Gilbert et al., 2001) and effectors (Inada et al., 2001). There are well-established beneficial therapeutic effects of TNF blockade on Crohn disease (Reimold, 2003) and there is a reported TNFα polymorphism at the *IBD3* locus that probably increases TNF production and is associated with some cases of ulcerative colitis and some cases of Crohn disease that have no *CARD15/NOD2* mutations (van Heel et al., 2002).

Interestingly, TNFα has recently been shown to be involved in the regulation of *CARD15/NOD2* expression (Rosenstiel et al., 2003). Whether or not the keratin mutations interfere with interactions between keratins and TNFα receptors, Fas or other keratin-interacting proteins remains to be seen.

The place of keratins in the etiology of IBD

The effects of these K8 mutations on keratin assembly are clear, and the data suggest multiple ways in which defects in keratin 8 (and/or K18) might predispose people to inflammatory bowel disease. Hypotheses on the mechanisms of human IBD are converging on a model of interdependent aspects of disease development. The most prominent characteristic of these disorders is a dysregulated or escalated inflammatory response, which is probably triggered by excessive entry of foreign antigens into the connective tissue underlying the intestinal epithelium, and leading to inflammation and tissue breakdown. Some candidate molecules involved in this inflammatory response arm of the disease have been implicated by genetics (de la Concha et al., 2000) and recently one gene has been identified convincingly as *CARD15/NOD2* (Hugot et al., 2001; Lesage et al., 2002; Ogura et al., 2001), mutated in 50% of patients in some populations with Crohn disease in the ileum (Ahmad et al., 2001).

The inflammatory aspect of the disease is, however, augmented, or possibly preceded (Irvine and Marshall, 2000), by increased permeability in the intestinal epithelium. No candidate genes have been identified to account for this increased transepithelial permeability, but this phenomenon could easily be explained by keratin mutations leading to loss of mechanical resilience in the cytoskeleton and thereby to an increase in epithelial cell fragility. There is now overwhelming evidence from fragility disorders in stratified squamous epithelia that mutations in keratins render epithelial cells fragile. If the mutations are only mild, as might be true of intestinal K8 mutations, or if the tissue is not subject to as much stress as the stratified epithelia, the body's healing process may normally be able to cope with this low-level fragility. Inflammatory bowel disorder may only arise if, in addition, the inflammatory response is incorrectly controlled.

Nevertheless, the number of patients with K8 mutations remains low, and no clear K18 mutations have so far been found in these patients. Thus, mutations in K8 or K18 are not a feature of all cases of IBD. However, there are likely to be many subtypes of IBD, if only one knew better how to classify them; it is already becoming clear from the *CARD15/NOD2* studies that IBD includes multiple diseases that are distinct at the molecular level. Keratin 8 is probably only one of many structural genes that could equally impact on the aetiology of IBD. As in the skin fragility disorders, defects in many other cytoskeleton or cell adhesion molecules can give rise to related phenotypes (discussed by Irvine and McLean, 2003). Cadherin mutations in intestinal cells of mice have already been shown to produce an IBD-like phenotype (Hermiston and Gordon, 1995). Taking all the cytoskeleton, junction or extracellular matrix genes together, there must be at least 20 genes mediating the structural resilience of intestinal epithelial cells.

In addition, different genetic or environmental factors could interact with the same keratin defects to produce different disorders of simple epithelia, such as cirrhosis of the liver, which is known to be associated with keratin 8 mutations (Ku et al., 2001; Ku et al., 1997) but was not observed among the IBD patients investigated here. Finally, genetic population differences in IBD patients are beginning to emerge, such as the absence of *NOD2/CARD15* mutations in Japanese and Chinese Crohn disease patients (Inoue et al., 2002; Leong et al., 2003; Yamazaki et al., 2002). Thus, keratin mutations may be associated with a specific subset of IBD patients that are not yet clinically distinguishable, and identification of these subsets may have important prognostic implications and could lead to useful diagnostic strategies and pre-emptive treatment.

This research work was funded by Cancer Research UK (C26/A1461 to E.B.L., supporting D.W.O., E.L.R., R.M.P.), with additional support from DEBRA (to E.B.L. supporting N.J.W.) and the Wellcome Trust [55090 to E.B.L. supporting A.J.M.H., 48687 to R.A.Q. supporting A.M.H. and a Clinician Scientist Fellowship (D.v.H.)]. Our thanks to Bishr Omary (Stanford University) for the K18 clone, to David Watts for assistance in sample collection, to Amar Al-Chalabi for some of the control samples and to Richard Currie for assistance with the surface plasmon resonance analysis. Finally, our thanks to Professor Peter Hall for the lively discussions that encouraged us to persist in this quest.

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