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Creating diseases to understand what prevents them: genetic analysis of inflammation in the gastrointestinal tract

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Abstract

Inflammatory bowel diseases (IBD), including both ulcerative colitis and Crohn's disease, are extremely variable in severity and have strong genetic components. In mice, several mutations are known to favor or inhibit intestinal inflammation. But a comprehensive picture of the pathogenesis of IBD cannot be assembled based on the limited information so far available from mouse genetic analyses, nor can human IBD be stringently ascribed to mutations known to be influential in mice. This review highlights recent progress made using mouse models created through a forward genetic approach towards the understanding of genes that normally prevent intestinal inflammation.

Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) are the two major forms of chronic inflammatory bowel disease (IBD) and are diagnosed based on clinical, pathological, endoscopic and radiological features [1]. UC is confined to the colon and is marked by shallow, bleeding, inflamed ulcerations of the mucosa. CD is a granulomatous disease, often transmural and sometimes marked by formation of fistulae to other organs or to the abdominal wall. It may occur anywhere along the length of the GI tract. Both UC and CD typically begin in the second or third decade of life, and a majority of affected individuals progress to relapsing and remitting chronic disease [2]. UC and CD are relatively common and show a wide range of severity.

IBD depends upon host immunity, and both innate and adaptive immune systems have been implicated [3,4]. Evidence from animal models indicates that failure to suppress immunity to the abundant intestinal foreign antigen load can cause inflammation. Maintaining the normal balance between competence to respond to intestinal pathogens and suppression of inflammatory responses to commensal microbes appears to depend on the integrity of the mucosal and epithelial barriers [5–7], proinflammatory signalling pathways (especially via NF- κ B) [8], and regulation of innate and adaptive immune responses in the intestine and draining lymphoid organs [9]. Disruption of any of these components has been shown to result in intestinal inflammation in animal models [2,4]. Defects in these components have also been implicated in human IBD, although fundamental knowledge of underlying pathogenesis remains poor. Even the most well-established causes of IBD, NOD2 mutations,

elucidate pathogenesis only in a subset of white patients with ileal Crohn's disease [2,4,10]. There is also evidence supporting a role for endoplasmic reticulum (ER) stress in intestinal inflammation (see below) [11,12••], and individual cytokines, notably TNF, are clearly important in IBD pathogenesis [13]. Monoclonal antibodies against TNF show a dramatic ameliorative effect in at least some CD and UC patients [14•].

Microbes

IBD also depends upon microbial flora within the gut. Microbes may either initiate or perpetuate the inflammatory response in IBD [15]. Antibiotics can be used in patients with CD as adjunctive treatment. The essential role of microbes in IBD is best documented in mice, since in strains prone to spontaneous chronic colitis, disease is entirely dependent on the presence of the luminal flora. Thus, colitis is not observed when any of these lines are maintained in a germ-free state, but rapidly emerges when they are reconstituted with bacteria that are considered normal components of luminal flora [4,16]. Certain bacterial strains might be pro-inflammatory or anti-inflammatory and therefore rational manipulation of the colonic flora of IBD patients might be a future treatment option. Bacterial species like *Klebsiella pneumoniae* and *Proteus mirabilis* have been shown to provoke colitis even in wildtype mice [17•,18]. On the other hand, *Bacteroides fragilis* protects mice from *Helicobacter hepaticus* induced colitis. This beneficial feature is mediated by the microbial molecule polysaccharide A.

Environment

Given the role of the microbiome in IBD causation, environmental factors strongly influence the development of IBD. For example, within a genetically homogenous Icelandic population, the incidence of CD increased sevenfold over a period of 45 years [19]. Only environmental or epigenetic factors could conceivably account for this, although it remains largely unknown which environmental factors are significant for disease development, with the exception of the use of tobacco [20]. Recent work in animal models suggests that viral infection (e.g. with norovirus) may play an important role in triggering inflammation in CD.

Genetics

IBD is a genetic disease, albeit with variable penetrance, probably owing to environmental factors, conceivably epigenetic factors as well. We know with certainty that IBD is heritable, because it runs in families [21,22], is observed at higher frequency within certain ethnic groups [23] and shows a high rate of concordance in identical twins [24–26]. The most fundamental questions about IBD concern its genetic etiology, since a clear understanding might provide new options for highly specific therapy. To date, most searches for causative mutations have been carried out in humans. Highly penetrant monogenic causes of IBD exist, but are rare. Homozygous null alleles of IL-10 and the IL-10 receptor have been shown to cause phenotypically classical CD [27•]. Because such examples of causation are hard to find, it is often stated that IBD is a complex genetic disorder. But while it clearly is a complex genetic disorder in some instances, it may have a monogenic etiology more often than we generally realize.

Genome scans carried out in several Caucasian populations around the world indicate that mutations affecting many genes are likely involved in predisposition to IBD, but these studies have been insufficient to establish cause and effect for any particular allelic variant of any individual gene. In fact, no mutation with an attributable relative risk higher than three has been discovered by the numerous scans reported so far [28]. Moreover, in aggregate, a high percentage of the genome is included within the genomic intervals implicated in these scans. On the other hand, it is necessary to keep in mind that whole genome association studies almost invariably miss certain types of genetic disorder. If

recent, dominant or semidominant mutations are responsible for the disease phenotype, they will go undetected. And because many loci are haploinsufficient or otherwise vulnerable to dominant mutation, and 1–2 coding changes occur *de novo* at each generation in humans [29], viable semidominant phenotypes of recent origin are common.

In several studies, a striking excess of heterozygous mutations has been identified within candidate genes by direct sequence analysis of a diseased population. In such cases, semidominant changes were presumed to cause phenotypes of interest, among them susceptibility to Gram-negative infection [30], hypertension [31], and hypercholesterolemia [32]. In each instance, a sizeable proportion of population-wide phenovariance was ascribed to changes in one or several genes with related function. The same approach may detect semidominant alleles of genes responsible for a considerable proportion of IBD.

On the basis of our observations in mice (see below), semidominant mutations affecting *MUC2* may have an etiological role in IBD. And more generally, we hypothesize that dominant deleterious alleles of numerous human genes may cause IBD.

At least some cases of IBD probably *do* reflect a complex genetic disorder: that is, a disorder caused by an unlucky combination of alleles at multiple loci. In the simplest case, two loci may be involved, but potentially there may be more than two. The conjoint effects of allelic variants at these loci are manifested as a disease. One likely example of a contributory locus is NOD2/CARD15, which was identified by several groups as a CD susceptibility gene based on classical genetic mapping studies in human populations [33,34]. However, while causally associated with CD, NOD2/CARD15 mutations are not sufficient for disease development. In the general population there are many more healthy individuals carrying the high-risk alleles than there are CD patients with the same polymorphism. Thus, even when NOD2/CARD15 mutations are present, pathogenic alleles at other loci must be combined with them in order for the disease phenotype to be expressed, or environmental or epigenetic effects must come into play [10,33].

In fact, there may be many different genetic etiologies of IBD, some monogenic and some polygenic. The ultimate challenge in IBD genetics is to identify all genes for which contributory pathogenic alleles exist.

Forward genetics as a means of defining the genetic target

To unveil new genes important for the pathogenesis of IBD, we have taken an unbiased, hypothesis-free approach, using germline mutagenesis to analyze the phenomenon in mice. We screen for mutations that cause susceptibility to IBD on a defined genetic background, using dextran sodium sulfate (DSS) as an environmental sensitizing agent. We reason that at a low dose, DSS delivers an insult to epithelial integrity comparable to many that occur in nature. When an exceptional mouse is found to develop severe colitis, we positionally clone the causative mutation, determine whether the phenotype is caused by an effect of the mutation on the epithelium or hematopoietic system, and form testable hypotheses concerning the participation of the validated gene in the disease pathogenesis. Approximately 6000 mice have been screened to date by placing them on 1% DSS in drinking water for a period of seven days. A total of 15 transmissible mutations causing hypersensitivity to DSS have been detected. Of these, five have been identified, and fall into four gene categories, concerned with sensing microbes, proliferation of epithelial cells, accommodating ER stress and packaging specialized proteins within secretory vesicles. The genes discovered in this way, and their role in colitis, will be discussed in brief in the following sections.

Muc2

Two mutant mice (*Schlendrian* and *Muskatenwein*) exhibited semidominant phenotypes, and were found to have allelic mutations in the MUC2-encoding gene [50]. The *Schlendrian* mutation converts cysteine 561 to phenylalanine in the D2 domain of MUC2, while the *Muskatenwein* mutation causes an isoleucine to asparagine change at position 2172 near the C-terminal end of the protein, between the C domain and the cysteine knot (CK) domain (Figure 1). Both mutations cause severe, semidominant susceptibility to DSS induced colitis and the *Schlendrian* mutant develops spontaneous colitis as well when mice reach several months of age. McGuckin and coworkers earlier demonstrated that excessive ER stress, resulting from misfolding of the highly expressed MUC2 protein in goblet cells, is responsible for the colitis phenotype of *Muc2* mutant mice [12••]. Several recent reports provide additional evidence for a causal role for ER stress in IBD.

Not only excessive ER stress, but an inadequate capacity for managing ER stress can also cause IBD. Mice lacking *IRE1b*, one of the key initiators of the unfolded protein response (UPR), are more susceptible to DSS-induced colitis [11]. Mice lacking *XBPI* in epithelial cells display a loss of secretory Paneth cells and goblet cells in the intestinal epithelia, as well as spontaneous inflammation in the ileum. Moreover, single nucleotide polymorphisms (SNPs) in the *XBPI* locus have been correlated with IBD in humans [35]. In addition, an ENU-induced hypo-morphic allele of the membrane bound site 1 protease (S1P)-encoding gene *Mbtps1* caused increased sensitivity to induced colitis. S1P is required for the ATF6-mediated UPR to occur, and this may also be essential for prevention of IBD when injury is caused by DSS [36].

Aqp3

The recessive *Phoebus* mutation [50] causes death within five days of continuous exposure to DSS. The *Phoebus* mutation is a T to C transition at position 202 of the *Aqp3* transcript resulting in a valine to alanine substitution at residue 43 of the aquaporin 3 protein (Figure 2). Aqua-porins mediate the rapid and selective transport of water across membrane lipid bilayers, allowing cells to regulate volume and internal osmotic pressure. AQP3 has an additional role in proliferation that has been reported for colonic epithelial cells, where it is expressed at the basolateral membrane [37]. Induction of colitis in AQP3 deficient mice by oral DSS or intracolonic acetic acid administration resulted in severe colitis after three days leading to death [37]. *Aqp3* mutations cause diabetes insipidus as well, and one mechanism behind the colitis phenotype in AQP3 deficient mice is increased daily fluid consumption and therefore the increased DSS dosage. However, AQP3 deficient mice also showed increased susceptibility to DSS colitis when the DSS administration was normalized according to the average daily water intake of each genotype group [37].

Enterocyte proliferation was greatly reduced in AQP3 null mice, presumably contributing to the reduced ability of these mice to cope with the damage induced by DSS. Oral glycerol treatment improved survival and reduced the severity of colitis in *Aqp3* null mice [37], suggesting that glycerol transport may be important for enterocyte proliferation during colitis.

Toll-like receptors

The *Liesgen* (aka *CpG7*) mouse [50] showed severe weight loss relative to wild type C57BL/6J mice and died after four days of continuous DSS administration. A G to T transversion was identified at position 1944 of the *Tlr9* transcript on chromosome 9. The mutated nucleotide results in conversion of an arginine to a leucine at residue 613 of the TLR9 protein, in the twentieth leucine rich repeat (LRR) of the TLR9 ectodomain. TLR9 has twenty-five such repeats (Figure 3). Several reports have established an important role

for TLR9 and other TLRs and components of the TLR signaling pathways in colitis. TLR2/3/4/5/9 and MyD88 deficient mice have a decreased tissue repair response during acute DSS damage [5,6,38–41]. We have recently demonstrated that TLR signaling plays an important role in nonhematopoietic cells, presumably cells of the gut epithelium, in which it induces biosynthesis of the EGF ligands amphiregulin (AREG) and epiregulin (EREG), triggering a protective response via EGFR in a model of DSS induced colitis [38]. A mutation in *Adam17* (*WavedX*) was found to cause hypersusceptibility to DSS as well, apparently because Adam17, a matrix metalloproteinase also known as TNF- α converting enzyme (TACE), is necessary for post-translational processing of EGF family ligands, including AREG and EREG [42]. Moreover, mutations of *Egfr* (the dominant *Velvet* allele) or *Areg* or *Ereg* strongly sensitize mice to DSS [38,43,44].

Yipf6

The recessive *Klein-Zschocher* phenotype, also detected in DSS screening, was ascribed to a splicing mutation in *Yipf6* (Ypt1p-interacting protein family member 6), a previously uncharacterized gene on the X-chromosome [45••]. The gene is predicted to encode a protein with a cytoplasmic N-terminal domain and a C-terminal domain containing five transmembrane α helices connected by short loops. Proteins of the Ypt1p-interacting protein 1 (Yip1) family are thought to regulate Rab protein-mediated ER to Golgi membrane transport (Figure 4). *Klein-Zschocher* mutant mice also develop spontaneous ileitis and colitis after sixteen months of age. Defective formation and secretion of large secretory granules by Paneth and goblet cells could account for impaired intestinal homeostasis in these mutant mice.

As Yipf6 is highly expressed throughout the gastrointestinal tract, particularly in the colon, individual Yip1 family members may have tissue-specific functions [46]. Because the null allele of *Yipf6* described here causes spontaneous disease, we suggest that *YIPF6* should be regarded as a susceptibility locus when searching for causes of IBD in humans, particularly males.

The general picture that emerges

When DSS disrupts the epithelial barrier, microbes gain access to the lamina propria and trigger a response leading to repair as schematically illustrated in Figure 4. This response is minimally dependent upon TLR signaling, and a nonredundant requirement for several different TLRs is evident. Mutations in genes encoding several of the TLRs, in *Unc93b1* (which permits TLR3, 7, 8, and 9 transport from the ER to early endosomes in which signaling occurs), in *MyD88*, and in other components of the TLR signaling apparatus will prevent repair. TLR signaling is principally important because it leads to upregulation of *Areg* and *Ereg*. Biosynthesis of these EGF family ligands and their processing by Adam17 must occur to allow signaling via EGFR. EGFR signaling stimulates the proliferation of epithelial cell precursors, permitting closure of gaps in the epithelium. Also essential in this process is the uptake of water molecules by rapidly dividing cells, and the management of ER stress, particularly in goblet cells, which produce enormous amounts of MUC2 protein, especially when stimulated to do so by signals that result from epithelial injury. ER stress can be overwhelming if missense mutations prevent proper folding of MUC2, or if mutations affecting the UPR pathway are present. In either case, cell death may occur, with failure to close epithelial gaps. Certain molecules released by Paneth cells are also critical for resolving injury. These could include antimicrobial peptides present in the large granules of these cells, which fail to form in *Yipf6* mutant mice. Absent such proteins the inoculum sustained during DSS injury may be too large for the host to cope with.

Conclusion

What mutations cause IBD? And what molecular processes normally prevent it? The former question has only a few answers. Monogenic causes of IBD have been elucidated in specific instances [27•], and many loci may be at risk of mutation. ENU mutagenesis would suggest that this is the case. Humans do not normally encounter DSS, but injury to the gut epithelium does occur on occasion, and it may be that some individuals are incapable of resolving it. It is interesting to see that when DSS is used in an unbiased sensitized screen, the mutations that cause severe colitis affect genes involved in epithelial repair. Could lesions in such genes be important causes of IBD in humans? As the list of such genes grows longer, DNA sequencing may provide a definitive answer.

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Figure 1.

Domain structure of Mucin2. The *Schlendrian* mutation causes a cysteine to phenylalanine change in the D2 domain of the MUC2 protein. The *Muskatenwein* mutation alters an isoleucine residue located near the C-terminal end of the protein between the C domain and the cysteine knot (CK) domain. S = Signal sequence; D = D domains (homology to VWF; mediates trimerization); CR = Cysteine-rich domain; TR = Tandem repeat domain (heavily O glycosylated); GDPH = GDPH autocatalytic proteolytic site; B = B domain (homology to VWF); C = C domain (homology to VWF); CK = Cysteine-knot domain (homology to VWF; mediates dimerization).

Current Opinion in Immunology

Figure 2.

Protein and domain structure of AQP3. The *phoebus* mutation is modeled on AQP1, for which a crystal structure is available. **(a)** Side view of crystal structure of bovine AQP1 [47] (PDB ID: 1J4N). Transmembrane helices (H1–H6) are shown in purple; two membrane-inserted, nonmembrane spanning helices, HE and HB, are in light orange. The residue corresponding to the *phoebus* mutation, as shown in [47], is colored green. **(b)** Top view of AQP1 looking down into the cytosol. Models created with PyMOL. **(c)** Domain structure of AQP3. The position of the *phoebus* mutation in H1 is indicated by a red asterisk. NPA, asparagine–proline–alanine motif.

Figure 3.

Protein and domain structure of TLR9. The *CpG7* mutation is modeled on the TLR3 ectodomain, for which a crystal structure is available. **(a)** Crystal structures of the mouse TLR3 ectodomain [48] (PDB ID: 3CIG) and human TLR2 TIR domain [49] (PDB ID: 1FYW). The residue corresponding to the *CpG7* mutation, as determined by BLAST alignment, is shown in orange. Model created with PyMOL. **(b)** TLR9 is a 1032 amino acid protein with an extracellular domain (purple) containing leucine rich repeats (LRR), a short transmembrane (TM) domain (pink) and a cytoplasmic Toll/Interleukin-1 receptor (TIR) domain (teal). The position of the *CpG7* mutation is shown.

Current Opinion in Immunology

Figure 4.

Schematic illustration of mutations causing susceptibility to intestinal inflammation. By screening mice with ENU-induced mutations with a low dose of DSS, we have identified a number of components necessary for protecting against intestinal inflammation. These include TLR signaling, epithelial growth factor receptor (EGFR) signaling, aquaporin 3 (Aqp3), the ER stress pathway that responds to unfolded proteins, and Yipf6, which is involved in formation and secretion of vesicles from Paneth and goblet cells. Allele names are in red; affected genes are in black.