New insights into the pathogenesis of Crohn’s disease: are they relevant for therapeutic options?

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Summary

During the last few years significant advances have been achieved in the understanding of the pathogenesis of inflammatory bowel disease (IBD). A genetic susceptibility to Crohn’s disease has been proven by identification of variations as risk factor NOD2/CARD15. Functional data on NOD2/CARD15 and NF-κB activation indicate that an inflammatory reaction of the intestinal mucosa, as an immediate response of the innate immune system, may be necessary for the maintenance of gut homeostasis. Crohn’s disease is now also discussed as an impaired and inadequate immune reaction and no longer only as a hyper-responsive of the mucosal immune system. Data on NOD2/CARD15 expression suggest that macrophages and epithelial cells could be the locus of the primary pathophysiological defect and that T-cell activation might just be a secondary effect inducing chronification of the inflammation, perhaps as backup mechanism to insufficient innate immunity. In addition to NOD2/CARD15 there are more “innate” pathways by which commensal and pathogenic bacteria can directly be hindered to invade the human body (such as interaction with Toll like receptors, TLRs and defensins). The “germ-concept” and the “genetic concept” of IBD pathophysiology are converging. However, more time is needed until these important insights in IBD pathogenesis will make their way into routine diagnostic procedures and treatment of patients with IBD.

Key words: inflammatory bowel disease; Crohn’s disease; pathogenesis; mucosal barrier; innate immunity

Susceptibility genes and IBD pathogenesis

Significant progress has been made in recent years to the field of IBD pathogenesis. A number of very important new insights have been gained. Whereas research on IBD pathogenesis used to be influenced by results from immunology and rheumatology, new findings in IBD patients stimulate other fields such as investigations on innate immunity and mucosal barrier functions. Studies on pathogenesis of Crohn’s disease (CD) have stimulated research in hematology (graft versus host disease), transplantation medicine (outcome of small intestinal transplantation), dentistry (periodontal disease) or pediatrics (immune deficiencies). It can be expected that this progress in the understanding of IBD pathophysiology will soon change our therapeutic concepts. Therefore, it will be important for physicians to know about those recent advances.

An involvement of genetic factors in CD pathogenesis had long been postulated. From twin studies it was evident that more that 50% concordance of CD in monozygotic twin pairs can be expected [1, 2]. A recent study again has shown concordance for CD in 63.6% among monozygotic twins, however, only 3.6% among dizygotic twins [3]. These data suggest that the genetic background is responsible for at least 50% of the risk or “susceptibility” to develop CD. Obviously, it is not a sufficient condition as otherwise there would be 100% concordance of disease in monozygotic twin pairs. On the other hand a genetic risk of 50% seemed to be promising enough to justify a worldwide search for susceptibility genes. It soon became obvious that not a single gene or mutation could account for the complete susceptibility to develop CD and that the pathogenesis of CD is based on a polygenic risk profile [1, 2].

The proof of the concept was achieved in 2001 with the discovery that NOD2/CARD15 is the most important susceptibility gene for CD [4–6]. About 20 to 40% of all patients – depending on
the genetic background – carry variants of this gene in contrast to 10–15% in the healthy population. In the previously mentioned study on monozygotic twins these relative amounts are even higher: 44% of patients with CD were positive for one or more mutant alleles of NOD2/CARD15 compared to 2% of UC patients and 19% of healthy twins [3]. By detailed analyses of the impact of the NOD2 gene knowledge has been gained about genotype-phenotype correlations but also about the difficulties in finding an impact of those discoveries for the clinical management of patients [7]. Three major NOD2 genetic variants are associated with CD in Caucasians in several independent studies. However, NOD2 variants are irrelevant in the Asian population and do not play any role for the pathogenesis of CD in Japan or China [8–13].

NOD2 variants explain up to one third of the genetic susceptibility for CD. Genotype-phenotype analyses have demonstrated some association of NOD2 variants with ileum-specific disease, an increased incidence of the fibrostenotic phenotype and an earlier age of disease onset [7, 14–17]. However, despite this, no other relationship between the NOD2 genotype and disease behavior or response to treatment could be identified so far – and there is no hope that there will be more definite relationships and associations identified in the future. Thus, the clinical impact of knowing the patient’s genotype is limited so far. Screening for NOD2/CARD15 mutations in order to identify high-risk individuals or to introduce an individualised disease management is therefore currently not recommended.

Many patients ask whether a genetic screening could not quantify the risk to develop CD in their children. The relative risk to develop CD is increased by a factor of 4–5, if NOD2/CARD15 variants are present. On the other hand, as previously mentioned, more than 10% of the healthy population carry NOD2/CARD15 variants. This already indicates that the absolute risk (which is the only important number) must be low: it increases from 0.1% to 0.4% if NOD2/CARD15 variants are present. Such numbers are not helpful for individual risk predictions – especially as there is no preventive treatment available.

What was learnt from the analysis of NOD2/CARD15 variants on CD pathogenesis?

The function of NOD2/CARD15 has been investigated in detail (see figure 1). NOD2/CARD15 was the first genetic base for stratifying disease phenotype locations and is associated with small intestinal involvement [18]. The most important information may be that it is an intracellular “alarm button”, a receptor recognising invading bacteria, that entered the mucosal wall. Muramyl dipeptide (MurNAc-L-Ala-D-isoGln, MDP), a component of the bacterial wall derived from peptidoglycan as the essential structure in bacteria, was found to be the major ligand for NOD2 [19, 20]. MDP is a component of the wall of Gram-positive bacteria. MDP is actively transported into epithelial cells via hPepT1, a brush border transporter expressed in the small intestine [21, 22] or it might be taken up via currently unknown mechanisms. NOD2 mutants associated with susceptibility to CD seem to be deficient in their recognition of MDP [20]. Interestingly, MDP was long known to be the essential structure in Freund’s adjuvants, important for vaccination success.

As NOD2/CARD15 confers the risk to develop CD in one third of patients, the question of which cells of the intestinal mucosa express NOD2/CARD15 was synonymous with the question of which cells are most relevant for the pathogenesis of the disease in those patients. Surprisingly expression was not found in those cells which are usually targeted with treatments, the central player of adaptive immunity: the T-lymphocytes (azathioprine, 6-mercaptopurine or methotrexate mainly act on T-cells). In contrast, NOD2/CARD15 protein expression was mainly found in the central cell component of the innate immune system, a cell type capable to phagocytose (eat and destroy) pathogens, to react to bacteria by secreting cytokines and toxic oxygen radicals: macrophages in the normal colon displayed the most prominent NOD2/CARD15 protein expression [23]. This surprising finding (which changed the view of IBD as a “T-cell disease”) was even “topped” by the results on NOD2/CARD15 protein expression in inflamed mucosa from CD patients. Again no expression in T-cells could be detected, however, increased NOD2/CARD15 expression was found in intestinal epithelial cells (IECs) and again macrophages in CD lesions [23–26]. A role for the epithelial Paneth cells in NOD2/CARD15 related IBD pathophysiology is supported by data showing NOD2/CARD15 mRNA enriched in crypts compared with villi, with Paneth cells being the most prominent cells expressing NOD2/CARD15 in normal and CD-mucosa [26–28]. Colonic epithelial cells also have been shown to express this protein, however, to lower mRNA levels as compared to Paneth cells or intestinal macrophages [25, 29, 30]. These data indicated that changes found in the “intestinal immune system” targeted by most therapies available so far are secondary. At least CD is not a “normal autoimmune disease” in which primarily T-cells are misdirected against “self-structures”. It
may even be assumed that the adaptive immune system does exactly what it is supposed to do. However, it receives misleading orders from a disturbed system of primary defense: the innate immune system, in the case of the intestinal mucosa consisting of the primary barrier forming epithelial cells and the intestinal macrophages. These exciting new insights can only be described as a change in the basic paradigm of IBD pathophysiology. They have changed the focus of big drug companies in their search for new therapeutic options for IBD, and this latter fact will certainly influence the upcoming therapeutic options for future treatment of IBD patients.

What is the normal function of NOD2/CARD15 and how is it changed during CD pathogenesis?

NOD2/CARD15 is a member of a superfamily of genes, the NBS-LRR proteins (for nucleotide-binding site and leucine-rich repeat), which are involved in intracellular recognition of microbes and their products [31]. NBS-LRR proteins are characterised by a C-terminal leucine-rich repeat (LRR) domain able to bind microbial motifs, an intermediary nucleotide binding site (NBS) essential for the oligomerization and signal transduction, and a caspase-activating and recruitment domain (CARD).

NBS-LRR proteins play an important role in the innate immune system. The family also includes such proteins as the so-called Nalp molecules. These proteins are involved in inflammatory responses and a number of auto-immune diseases are related to mutations in these family members [32]. There is a second class of “detection molecules” for bacterial and viral products, which are named toll like receptors (TLRs). Both classes of microbial product sensors are classified as “pattern recognition receptors” (PRRs). After being activated by the presence of microbial ligands they usually initiate a defense response. The microbial ligands of PRRs have been termed “pathogen-associated molecular patterns” (PAMPs), however, not all molecules detected are always pathogenic. For example, bacterial or viral DNA motifs bound by TLR9 may induce amelioration of colitis as well as aggravation depending on the circumstances [33–35].

The microbial patterns recognised by PRRs are evolutionary highly conserved. Plants already have an innate immune system of PRRs recognising the same patterns of microbes as compared to humans. This indicates several important points: i) the innate immune system has been developed very early in evolution and obviously has been very successful as it is highly conserved. ii) as it is likely to be very important for self defense its expression is most relevant at sides of high antigen and pathogen density, such as the intestinal mucosa. iii) disturbances of these functions are likely to cause diseases. As the function is very basic different alterations may finally lead to the same reaction pattern: mucosal inflammation.
In summary, both classes of PRRs (NLRs and TLRs) are involved in detecting potentially harmful microbes through PAMP recognition followed by the initiation of a defense reaction and sometimes but not always inflammation with activation of the adaptive immune system. Defense reactions beside activation of the adaptive immune system may be the secretion of locally acting antibacterial molecules such as oxygen radicals [36].

NOD2/CARD15 induced signal transduction is usually followed by NF-κB activation. Mutations of the gene, as found in CD, are thought to be associated with an impaired activation of NF-κB.

MDP – NOD2/CARD15 interaction is followed by activation of the innate immune system reflected by an induction of α- and β-defensins secretion as a first line of defense at the mucosal barrier in response to a bacterial attack. Nod2 protein activation furthermore increases the production of pro-inflammatory cytokines such as TNFα, IL-1β or IL-8, which also reflects an early defense mechanism [29, 37–39].

In epithelial cells MDP binding to NOD2 is specifically followed by an induction of the expression of the inducible antimicrobial peptide hBD-2 [38]. The hBD-2 promoter contains putative binding sites for NF-κB providing an explanation how NOD2 activation may induce hBD-2 transcription (fig. 2) [38]. Mutation of the two proximal NF-κB sites in the hBD-2 promoter region almost completely inhibits the MDP-induced hBD-2 promoter activation in NOD2-overexpressing cells [38].

**Nod2 variants are associated with reduced production of defensins**

Analyses of CD Patients with wild type (wt) Nod2 showed that the expression of human α-defensin 5 (HD5) is approximately 50% reduced in NOD2 variant subjects when compared to wt patients [40]. HD6 levels were similarly reduced. In contrast no significant changes in most other Paneth cell antibacterial factors were found, suggesting a specific defect of α-defensin production associated with NOD2 variants.

In a parallel study, the same group found in general, normal levels of β-defensins in CD patients whereas there were increased levels of β-defensins 2 and 3 in UC patients [41]. Therefore, Wehkamp and colleagues suggested that in CD there is also a lack of β-defensin induction and thus a relative deficiency of this defensin contributing to impaired barrier functions.

One of the first findings indicating a role of Nod2 for intestinal barrier function was the discovery that Nod2 is involved in the regulation of α-defensin expression. As previously mentioned, Nod2 is expressed in Paneth cells and protects epithelial cells from bacterial infection. Patients carrying the SNP13 variant (a frameshift mutation at Leu1007) were identified to exhibit the most severe decrease in mucosal HD5 levels when compared to Nod2 wt CD patients [40, 42]. Patients with CD, not carrying this mutation also show a decrease of defensins, which is caused by altered WNT signalling (WNT TCF4) [43].

Nevertheless it is not unequivocally proven that the reduction in defensin production and subsequent deficiency in antibacterial activity caused by Nod2 variants (or at least one of the three major Nod2 variants) is a major factor in the pathogenesis of CD. An impairment of mucosal barrier function can itself be a cause of gut inflammation. A chimeric mouse expressing a dominant-negative N-cadherin transgene in the intestinal epithelium, followed by leaky tight junctions between cells, developed severe mucosal inflammation [44].

**As the detection of microbial antigens is a basic function of the mucosal immune system: Is it only relevant for CD?**

As mentioned above a disturbed innate immune response is a very important and basic mechanism of self defense and should not only be relevant for CD. Therefore, it was only a short time until the impact of the new insights into CD pathogenesis for other diseases could be demonstrated. One of the first fields in which a high impact of NOD2 variants on disease development, pathogenesis and even disease associated mortality could be demonstrated was graft versus host disease (GvHD) after allogeneic bone marrow transplantation.

Individuals suffering from intestinal GvHD after allogeneic bone marrow transplantation (SCT) show histological features similar to CD. GvHD is associated with increased intestinal permeability and could therefore also be a problem of a defective intestinal barrier. It is still the most severe complication following SCT. Experimental models indicate the primacy of gastrointestinal damage: Conditioning related damage of the intestinal epithelium results in bacterial translocation followed by increased cytokine release by macrophages/monocytes and T cell activation [45, 46].
The incidence of severe GvHD (and associated gastrointestinal GvHD) rose from 18% in donor/recipient pairs without any NOD2/CARD15 variant to 37% in pairs with either donor or recipient mutations with a subsequent increase of treatment related mortality (TRM) from 33 to 60% [47–49]. In a subgroup of 11 donor/recipient pairs where both donor and recipient had NOD2 variants, severe GvHD rose from 22 to 55% and transplantation related mortality rose from 38% to 100% [47–49].

A deficient antibacterial response with decreased ability to clear commensal bacteria in both, IEC/paneth cells of the recipient’s mucosa and donor monocytes might result in increased bacterial translocation and subsequent mucosal inflammation in this case [42]. Assuming a comparable pathophysiology in GvHD and CD, these data again support the hypothesis that the primary pathophysiology in a subgroup of CD patients is a IEC- and monocyte/macrophage defect and that alterations in T-cell function are secondary.

As the stem cell donors also seemed to have a major impact, further conclusions can be drawn for NOD2/CARD15 functions on the intestinal barrier. A NOD2/CARD15 variant mediated altered pathway of activation of intestinal macrophages or antigen presenting cells (APCs) might be an additional important mechanism that could at least explain the strong association of NOD2/CARD15 variants with GvHD. When the causes of death in the investigated SCT-patient cohorts were analyzed, GvHD and progressive pulmonary failure resembling adult respiratory distress syndrome were the major causes of death in recipient/donor pairs with NOD2/CARD15 variants [47, 49]. As APCs express NOD2/CARD15, the altered pathways of APC activation might not only be relevant for the intestinal barrier but also involve other organs forming a barrier against the exterior, such as the lung.

Other genetic polymorphism and alterations found to be involved in the pathogenesis of CD

A number of other genetic polymorphisms have been reported to play a role in IBD pathogenesis. The human multidrug resistance 1 (MDR1) gene product P-glycoprotein is highly expressed in intestinal epithelial cells and constitutes a barrier against xenobiotics. Polymorphisms causing lower protein expression have been associated with the risk to develop UC or IBD in general [50, 51]. Therefore, P-glycoprotein could play a role in the defense against intestinal bacteria [52].

In a genome-wide association study, 19779 non-synonymous single nucleotide polymorphisms were investigated in 735 individuals with CD and 368 controls [53]. The authors found a disease association of rs2241880 in the autophagy-related 16-like 1 gene (ATG16L1) which could be replicated. The ATG16L1 gene encodes a protein in the autophagosome pathway that processes intracellular bacteria. The authors also found a statistically significant interaction with respect to CD risk between ATG16L1 and the NOD2/CARD15 susceptibility variants [53]. This gene and its protein product makes the autophagic pathway an attractive therapeutic target and drug companies have started developing treatment strategies aimed at this gene. A second study analysing the ATG16L1 gene in human and mouse intestinal Paneth cells found very similar results, potentially linking this gene to defensins [54].

A genome-wide association study of ileal Crohn disease and two independent replication studies identified strong and significantly replicated associations with a coding variant in ATG16L1 [55]. They also found strong associations of variations in the genomic regions encoding PHOX2B, NCF4 and a predicted gene on 16q24.1 (FAM92B) [55]. The authors demonstrated that ATG16L1 is expressed in intestinal epithelial cell lines and that functional knockdown of this gene abrogates autophagy of Salmonella typhimurium. Again these results confirmed that host cell responses to intracellular microbes (involving autophagy) are crucial in the pathogenesis of CD.

In a genome-wide association study of 3230 CD cases and 4829 controls (all of European descent) the Welcome Trust consortium could confirm eleven associations previously replicated and established at genome-wide significance levels including NOD2, 5q31 (IBD5) as well as IL23R, ATG16L1, IRGM, TNFSF15 and PTPN2 [56]. In addition 21 new loci could be replicated with newly IBD associated genes such as PTPN22, TTN1, IL12B, CDKAL1, CCR6, JAK2, C11 or f30, MUC19 or STAT3 [56]. The function of a number of those genes is currently unclear and is still to be defined [57]. At this time the identified variations explain only a fraction of IBD burden in the population, suggesting that other factors, such as interacting environmental factors, are major contributors to disease susceptibility [58].

Furthermore, a reduced gene copy number of the β-defensin cluster on chromosome 8 results in an attenuated induction of human β-defensin 2 (HBD2) and is associated with colonic involvement of CD [59].
“Let them eat dirt”: The role of bacteria and probiotics

With the finding, that most susceptibility genes for CD are involved in innate immune mechanisms and the primary defense against bacteria entering the mucosa, for the first time a unifying concept of the “genetic pathophysiology hypothesis” and the “environment pathophysiology hypothesis” of IBD was possible. Bacteria are the link between environment and mucosal defense system.

However, evidence that bacteria play a major role in the initiation and perpetuation of intestinal inflammation has been obtained long ago in animal studies with germ free maintained mouse models, a condition under which these animals do not develop intestinal inflammation in contrast to specific pathogen free (SPF) kept rodents [60].

In colonic lesions of CD patients adherent-invasive E. coli have been found. In addition an increased bacterial translocation into deeper layers of the mucosa has been described in CD patients, which could be of pathophysiological relevance. E. coli Nissle has been proven to be of therapeutic potential in IBD [61–64]. The mechanism could be an inhibition of the adherence and invasion of pathogenic E. coli [65], which further supports a role of bacterial translocation into the mucosa in the pathogenesis of CD. In fact fecal bacterial composition is altered in CD patients compared to healthy persons [66].

A role for certain bacteria in the pathogenesis of IBD is further supported by the positive effects of probiotic bacteria on intestinal inflammation, secretion of pro-inflammatory cytokines and induction of β-defensins [67–76]. An increased bacterial invasion into the mucosa could be caused by ineffective innate responses such as mutated and defective NOD2. On the other hand impaired or defective protection mechanisms of the mucosa could be involved.

A direct mucosal protection is mediated by molecules such as mucins, trefoil peptides or defensins. A deficiency in these molecules could cause a breakdown of mucosal protection [37, 43, 77–80].

In humans, Swidsinski and co-workers demonstrated that the intestinal mucosal surface beneath the mucus layer is usually free of bacteria. In patients with CD or UC, the thickness of the mucosa-protecting mucus layer is decreased. More importantly, bacterial adherence to the epithelial surface could be demonstrated as well as epithelial tissue defects and deep mucosal infiltration with bacteria and leucocytes [81, 82]. The mucus above the epithelial cells is strongly colonised with bacteria in biopsies from patients with ulcerative colitis and Crohn’s disease when compared to controls [81 and own unpublished data]. This indicates that the epithelial mucus layer normally prevents contact between luminal bacteria and epithelial cells. However, in contrast it indicates that IBD is associated with breaks in the mucus barrier and colonisation of the mucus with bacteria.

Those bacteria colonising the mucus may be able to directly influence intestinal barrier integrity [83–85].

Conclusion

The genetic knowledge of IBD is increasing and turns out to be a success story. As more candidate genes become available, it is likely that the main gene variants predisposing to IBD will be found. As a whole the published literature indicates that Nod2 mediated NF-kappaB activation, subsequent induction of anti-microbial peptides such as defensins and the induction of cytokine expression are essential for the function of the intestinal barrier and for the prevention of bacterial translocation. The data indicate why a defect in the induction of this acute defense response is associated with chronic inflammation, as invading bacteria that cannot be readily detected and eliminated may start a backup mechanism of inflammation, finally resulting in chronic inflammatory reaction, followed by further impairment of the mucosal barrier. These new insights in the pathophysiology of CD will provide further understanding of concepts such as environmental influence, smoking, diet and intestinal flora in the modulation of CD susceptibility. Hopefully, the translation of genetics to clinical benefit will soon become reality.

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