The effects of NOD2/CARD15 mutations on the function of the intestinal barrier

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Abstract

NOD2 variants have been identified to be a susceptibility factor for Crohn’s disease. The NOD2 protein is an intracellular sensor of the bacterial wall product muramyl dipeptide (MDP) and activates the transcription factor NF-kappaB upon MDP-binding. NOD2 variants are associated with reduced NF-kappaB activation and reduced production of epithelial derived antibacterial peptides such as defensins. A reduced expression of defensins is described and found in patients with Crohn’s disease and ulcerative colitis especially when NOD2 variants are present. Furthermore recent evidence from mouse models suggests that the ability of intestinal epithelial cells to activate NF-kappaB upon bacterial stimulation protects from mucosal inflammation.

Taken together these data indicate that NOD2 mediated NF-kappaB activation, subsequent induction of anti-microbial peptides such as defensins and 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. 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.

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1. Introduction

Genetic factors have been postulated to be involved in the pathogenesis of IBD. The search for "risk genes" had an initial success in 1996 when the first susceptibility locus for Crohn’s disease (CD) was identified in the pericentromeric region of chromosome 16 and later named "IBD1." In 2001 a CAspase Recruitment Domain containing protein NOD2/CARD15 was found to be mutated in 20–30% of CD patients making it the proof of principle of the "genetic concept" of IBD pathophysiology. Multiple mutations in the NOD2/CARD15 gene have been identified, three of which have been shown to be independently associated with CD (arg702trp, gly908arg, and leu1007fsinsC). NOD2/CARD15 mutations are associated with ileal disease, earlier onset of disease and stricturing disease.

2. NOD2/CARD15 expression in the intestinal mucosa

NOD2/CARD15 is mainly expressed in the cytoplasm of macrophages and other mononuclear cells (antigen presenting cells) in the colon. In the ileum strong additionally expression is found in Paneth cells. But also colonic epithelial cells have been shown to express this protein, however, to lower mRNA levels as compared to Paneth cells or intestinal macrophages. Increased NOD2/CARD15 is found in intestinal epithelial cells (IECs) and macrophages in CD lesions. NOD2/CARD15 mRNA and protein expression are induced by pro-inflammatory factors such as tumour necrosis factor alpha (TNFalpha). Another regulator of NOD2/CARD15 gene expression present in increased amounts in CD mucosa is interferon gamma (IFNγ). This indicates that inflammation may induce increased levels of NOD2/CARD15 protein in IECs as well as intestinal macrophages (IMACs). In normal mucosa IMACs are in a functional tolerogenic and anergic state. They cannot be stimulated by bacterial products such as LPS as they show absent or decreased expression of a number of relevant receptors. However, in CD patients they display these receptors allowing activation and secrete large amounts of pro-inflammatory cytokines. This could be followed by induction of NOD2/CARD15 expression in IECs and – in an autocrine pathway – IMACs.

3. NOD2/CARD15 is a pattern recognition receptor (PRR)

NOD2/CARD15 is a member of a superfamily of genes, the NBS-LRR proteins (nucleotide-binding site and leucine-rich repeat), which are involved in intracellular recognition of microbes and...
their products and include Apaf-1 and NOD1/CARD4. NBS-LRR proteins are characterized by a C-terminal leucine-rich repeat (LRR) domain able to sense a microbial motif, an intermediary nucleotide binding site (NBS) essential for the oligomerization and signal transduction and a caspase-activating and recruiting domain (CARD) (Fig. 1). In contrast to NOD1, NOD2 has two CARD domains, which are also found in most caspases (Fig. 1). They form a three-dimensional structure that is very similar to the death domains of apoptotic related proteins. The CARD domains mediate protein–protein interaction through homophilic binding (Fig. 1).

NBS-LRR proteins play an important role in the innate immune system. The family also includes such proteins as Nalp molecules and some transcriptional regulator such as IPAF. Most of the mentioned proteins are involved in inflammatory responses and a number of auto-immune diseases are related to mutations in these family members. There is a second class of “detection molecules” for bacterial and viral products, the so-called toll like receptors (TLRs) (Fig. 1). 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 defence 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 TLR-9 may induce amelioration of colitis as well as aggravation depending on the circumstances.

The microbial patterns recognised by PRRs are evolutionary highly conserved. Both classes of PRRs are involved in detecting potentially harmful microbes through PAMP recognition followed by the initiation of a defence reaction and sometimes but not always typical inflammation with activation of the adaptive immune system. Defence reactions beside activation of the adaptive immune system may be the secretion of locally acting antibacterial molecules such as oxygen radicals or defensins (see below).

In the far most situations the intestinal mucosa will not be confronted with just one specific single PAMP but with an entire microbe or a number of different microbes exposing the mucosa to a multitude of different PAMPs at the same time. Therefore, it is likely that we will have to deal with typical “PAMP-patterns” to learn more about defence reactions and barrier functions of the intestinal mucosa in the future. NOD2 (similar to NOD1) recognises peptidoglycan (PGN), although both detect different motifs within this structure. PGN is the major constituent of the cell wall of Gram-positive bacteria. In Gram-negative bacteria it is found in a thin layer in the space between the outer and cytoplasmic membranes. The NOD2 protein has been found to bind and recognize muramyl dipeptide (the minimal motif in all PGNs) mediating consecutive NF-κB activation (see below).

4. NOD2/CARD15 induced signal transduction

After ligand binding the NOD2 protein associates with a serine/threonine kinase called RIP2 (also known and RICK or CARDIAD) through a homophilic CARD–CARD interaction (Fig. 1). This followed by the activation of RIP2 by a mechanism called “induced proximity” involving oligomerization and further activation. Activated RIP2 then interacts with the regulatory subunit of the IKK complex, IKKγ or NEMO (Fig. 1). Subsequent phosphorylation of IκBα, degradation of IκBα and release and nuclear translocation of NF-κB occur.

NF-κB is known to induce the expression of pro-inflammatory mediators such as TNF or IL-1 as well as other cytokines, chemokines, adhesion molecules or many more. NF-κB is activated after binding of ligands to various PRRs such as NOD proteins and Toll like receptors (TLRs).

Variants of the NOD2/CARD15 gene as found in CD are associated with an impaired activation of NF-κB in vitro in transfection experiments. The frameshift variant, Leu1007fsinsC, truncates the LRR domain and is associated with a markedly reduced NF-κB activation. In comparison, the Arg702Trp and Gly908Arg variants respond more to stimulation with bacterial wall products than the Leu1007fsinsC variant, however, the ability to activate NF-κB is still significantly reduced.

These data indicate that a reduced ability to activate NF-κB in response to intestinal bacteria may trigger CD. This is in contrast to the paradigm of a primarily hyper-responsive mucosal immune system being the cause of CD. As NF-κB is activated by pattern recognition receptors such as TLRs or NOD2 and NOD2 variants lead to defects in innate immune and epithelial barrier functions it was speculated that inhibition of NF-κB might also be associated with impaired innate immunity. Indeed evidence for a protective role of NF-κB has been found. Recently it was demonstrated that intestinal epithelial cell-intrinsic Ikkγ-dependent gene expression is a critical regulator of responses of dendritic cells and T cells in the intestinal mucosa. Mice with an epithelial-specific deletion of Ikkγ showed a reduced expression of the cytokine lymphopoietin in the intestinal mucosa and, after infection with the parasite Trichuris, failed to develop a pathogen-specific Th2 response associated with persisting infection. In addition these animals showed increased production of interleukin-12/23p40 and TNF leading to severe intestinal inflammation. Further evidence was found for an important role of NF-κB for intestinal epithelial integrity and the interaction between the mucosal immune system and gut microflora. In a mouse model of epithelial-cell-specific inhibition of NF-κB through a epithelial-specific deletion of NEMO severe chronic intestinal inflammation occurred associated with increased apoptosis of colonic epithelial cells, impaired expression of antimicrobial peptides and translocation of bacteria into the mucosa. Deficiency of MyD88 is associated with a lack of TLR induced signalling (see Fig. 1). MyD88 deficiency prevented the development of intestinal inflammation indicating that TLR-ligation is essential for disease pathogenesis in this mouse model.

These findings demonstrate that a complete lack of PRR (or PAMP respectively) induced NF-κB activation in intestinal epithelial cells is associated with mucosal inflammation. NF-κB activation may be essential for epithelial cell protection and innate defence mechanisms especially in a state of acute challenge such as bacterial translocation. In this situation an acute inflammatory response might be necessary to eliminate the invading microbiota.

Besides NF-κB induction, NOD2 has been shown to be involved in the induction of apoptosis. NOD2 may interact with the CARD domain of caspase 9 and potentiate an apoptotic response induced by other triggers. Evidence for an involvement of NOD2/CARD15 into epithelial cell apoptosis has been
derived from overexpression experiments thus not directly allowing conclusion for the in vivo situation. In CD patients a loss of the potentiation of apoptosis by the presence of NODs/CARD15 variants could be followed by an altered ability to undergo regular apoptosis and anoikis which could favour the development of necrosis. This might result in the generation of ‘danger signals’ promoting the induction of inflammation and an impairment of the epithelial barrier. Necrosis of epithelial cells could also be relevant in the pathogenesis of ulcers.

5. Anti-microbial peptides and the mucosal barrier function

The intestine is a reservoir of bacteria and of bacterial products (endotoxins, exotoxins, and cell wall fragments) that may escape from the intestinal lumen to the mesenteric lymph nodes and the bloodstream. The intestinal mucosa is the major barrier against those bacteria and toxins, protecting the body from the potentially harmful pathogens. Under physiological conditions the integrity of the intestinal mucosal barrier is maintained by a combination of mechanical, biochemical, and immunological mechanisms. The mechanical barrier is formed primarily by the epithelial cells and by their intercellular junctions. They also are part of innate immune mechanisms. Alterations in one these components of the intestinal barrier have been reported to be responsible for bacterial and endotoxin translocation. As discussed the cells of the intestinal mucosa express a number of PRRs which detect microbial components extra- or intra-cellularly.

A component of the biochemical defense mechanisms of the intestinal barrier is the luminal secretion of molecules, such as defensins and cathelicidins that directly destroy microorganisms. In mammals, defensins are the predominant anti-microbial polypeptides. Defensins have the capability to kill and/or inactivate a large spectrum of bacteria, fungi, and viruses. Six β-defensins (hBD-1 to hBD-6) have been identified in humans. hBD-1 is constitutively expressed, whereas hBD-2 and hBD-3 can be induced by microbial products and cytokines, such as IL-1 and TNF. Thus, β-defensins play an important crucial role for the barrier function of the intestinal mucosa especially as they are inducible upon challenge.

6. NOD2 activation and production of antimicrobial peptides

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 (Fig. 2), which also reflects an early defense mechanism.

In epithelial cells MDP binding to NOD2 is specifically followed by an induction of the expression of the inducible antimicrobial peptide hBD-2. The hBD-2 promoter contains putative binding sites for NF-κB providing an explanation how NOD2 activation may induce hBD-2 transcription (Fig. 2). 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.

Another protein important for intestinal barrier function which also seems to be modulated in its expression by NOD is...
"deleted in malignant brain tumors 1" (DMBT1). DMBT1 belongs to the group of secreted scavenger receptor cysteine-rich proteins and is considered to be involved in host defense by pathogen binding. Intestinal epithelial cells up-regulate DMBT1 expression upon proinflammatory stimuli (e.g., TNF, LPS). DMBT1 inhibits cytoinvasion of *Salmonella enterica* and LPS- and muramyl dipeptide-induced NF-κB activation and cytokine secretion in vitro. Following MDP mediated activation of NOD2, expression is DMBT1 is strongly up-regulated. Increased expression is found in the inflamed intestinal mucosa of Crohn’s disease patients with wild-type, but not with mutant NOD2. Dysregulated intestinal DMBT1 expression due to mutations in the NOD2/CARD15 gene may be another mechanism responsible for the dysfunction of the intestinal barrier in CD.

7. 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% decreased as compared to controls. 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. In a parallel study, the same group found normal levels of β-defensins in CD patients whereas there were increased levels of β-defensins 2 and 3 in ulcerative colitis (UC) patients. 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 again overall contributing to impaired barrier functions.

One of the first findings indicating a role of NOD2 for intestinal barrier function was the finding that NOD2 is involved in the regulation of α-defensin expression. As mentioned above NOD2 is expressed in Paneth cells and protects epithelial cells from bacterial infection. Mutations in NOD2/CARD15 have been demonstrated to affect α-defensin production in patients with CD. Patients carrying the so called SNP15 variant (a frameshift mutation at Leu1007) were identified to exhibit a further decrease in mucosal HD5 levels than that seen in NOD2 wt CD patients.

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.

Figure 3  NOD2 mediates NF-κB activation in epithelial cells which is followed by an induction of anti-microbial peptides such as defensins and induction of cytokine expression. This mechanism is essential for the function of the intestinal barrier and for the prevention of bacterial translocation. If NOD2 variants are present a defect of this acute defense response occurs followed by a leaky barrier, bacterial invasion and subsequent chronic inflammation finally further impairing the mucosal barrier.
Further arguments are needed to connect NOD2 variants with impaired barrier function of the intestinal mucosa.

8. NOD2/CARD15 and barrier function

Intestinal epithelial cells expressing mutated NOD2 are not able to respond appropriately to an in vitro challenge with Salmonella.\(^{46}\) The survival of Salmonella typhimurium in human intestinal Caco-2 cells was shown to be altered by the expression of NOD2.\(^{27}\) Nod2-deficient mice exhibit lower expression levels of cryptidins, the murine orthologs of human \(\beta\)-defensins, and exhibit a higher susceptibility to infection with Listeria monocytogenes.\(^{36}\) NOD2 mediates intracellular bactericidal activity possibly via interaction with GRIM-19, a protein with homology to the NADPH dehydrogenase complex.\(^{67}\)

Increased intestinal permeability has long been described in patients with CD.\(^{68}–72\) Clinical investigation of large pedigrees has suggested that the pattern of increased permeability in families with CD follows an autosomal recessive mode of inheritance. The increased permeability in CD is associated with the presence of NOD2 variants.\(^{73}–74\) These findings suggest that indeed NOD2 variants could be the underlying genetic defect that causes a defect of intestinal barrier functions (Fig. 3).

9. Epithelial barrier and graft versus host disease

Further arguments for the crucial role of NOD2 for intestinal barrier function besides its role in the pathophysiology of CD are findings in a completely different group of patients. Individuals suffering from intestinal graft versus host disease (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.\(^{75}–76\)

The incidence of severe GvHD (and associated gastrointestinal GvHD) rose from 18% in donorrecipient pairs without any NOD2/CARD15 variants to 37% in pairs with either donor or recipient mutations with a subsequent increase of treatment related mortality (TRM) from 33 to 60%.\(^{77}–79\) When donorrecipient pairs both had NOD2/CARD15 mutations severe GvHD rose from 22 to 55% and transplantation related mortality from 38% to 100%.\(^{77}–79\)

As the stem cell donors also seemed to have a major impact further conclusion can be drawn for NOD2 functions on the intestinal barrier: A NOD2 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.\(^{78}–79\) As APCs express NOD2, 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.

A deficient antibacterial response in both, IEC/Paneth cells of the recipient’s mucosa and donor monocytes might result in increased bacterial translocation and subsequent mucosal inflammation. Assuming a comparable pathophysiology in GvHD and CD, these data suggest that the primary pathophysiology in a subgroup of CD patients is an IEC — and monocyte/macrophage defect in bacterial product recognition and that alterations in T-cell function may be secondary.

References

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