

Inflammatory bowel disease

NOD2/CARD15 and the Paneth cell: another piece in the genetic jigsaw of inflammatory bowel disease

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Expression of NOD2/CARD15 in the Paneth cell may be critical in the pathogenesis of Crohn's disease

The emergence and application of novel molecular techniques over the last decade has provided a needed catalyst to studies of the pathogenesis of the chronic inflammatory bowel diseases (IBD): Crohn's disease (CD) and ulcerative colitis (UC). Successful development of genetically engineered models of intestinal inflammation has not only provided insight into the dysregulation of the mucosal immune system characteristic of IBD but has also emphasised the critical and complex role of the bacterial flora in establishing and maintaining chronic intestinal inflammation.¹ These advances in understanding pathophysiology in turn have already led to novel therapeutic approaches.^{2,3}

However, it is in studies of human genetics that landmark progress has been made, widely recognised not only within gastroenterology but also by investigators in all complex diseases.⁴ Genome wide scanning led initially to the identification of a number of susceptibility loci, the statistical evidence for which satisfy stringent criteria for definite linkage.⁵ The subsequent detection of the NOD2/CARD15 gene⁶⁻⁸ within the IBD1 linkage interval and the association of mutations within this gene with susceptibility to CD is widely regarded as the most stringent proof of principle for hypothesis free genome scanning in complex diseases.

In the time that has elapsed since the discovery of NOD2/CARD15, the contribution of this gene in determining susceptibility and disease behaviour in IBD has received detailed examination. It is now clear that NOD2/CARD15 mutations are associated with susceptibility to CD but not UC.⁶ However, the contribution is subject to considerable ethnic and even regional variation. Whereas mutations may be carried by up to 50% of central Europeans with CD,⁹ these mutations are not present in Japanese¹⁰ or Afro-American¹¹ patients. Even within Europe, there is considerable regional variation¹²⁻¹⁵ and reported

population attributable risks vary from 7.1% to 32%. Furthermore, there is heterogeneity within CD, and genotype-phenotype relationships clearly exist. Independent data suggest that NOD2/CARD15 variants are associated with early onset disease,^{9,11} involvement of the terminal ileum,^{16,17} and fibrostenosing disease,¹⁸ all phenotypic characteristics initially implicated in Crohn's initial descriptions of regional enteritis.¹⁹

The most intriguing question that remains to be answered concerns the mechanism whereby mutations in the NOD2/CARD15 gene predispose towards the chronic intestinal inflammation characteristic of CD. Studies with respect to protein structure, expression, and function promise to provide the answers but the critical questions remain unresolved. The NOD2/CARD15 gene, initially described by Ogura and colleagues,²⁰ encodes a 1040 amino acid protein, a member of the Apaf1/CARD family of cytosolic proteins, involved in apoptosis (programmed cell death). NOD2/CARD15 has sequence homology with other family members,²¹ notably NOD1/CARD4, which itself is not associated with CD susceptibility.²² The gene comprises two N terminal caspase activation and recruitment domains (CARD), a nucleotide binding domain, and a C terminal sequence of leucine rich repeats (LRR). The majority of CD associated mutations directly affect the LRR, which is a motif common to bacterial resistance R proteins in plants and mammals, notably the Toll-like receptor family,²³ enabling recognition of pathogen associated microbial patterns (PAMPs). Following PAMP recognition, the NOD2/CARD15 proteins dimerise and an interaction with the serine-threonine kinase RICK in the cytosol occurs, triggering downstream nuclear factor κ B (NF κ B) activation.²⁰ The original expression studies had suggested that NOD2/CARD15 was expressed only in monocytes, and implicated the protein as an intracellular regulator of NF κ B activity, sensitive to

bacterial lipopolysaccharide (LPS), complementary to NOD1/CARD4.²⁰

In recent months strong scientific evidence has emerged to complement initial observations. It is now clear that NOD2/CARD15 expression occurs not only in monocytes but may also be induced in dendritic cells²⁴ and intestinal epithelial cells.²⁵ Furthermore, independent data suggest that the minimal bacterial motif recognised by NOD2/CARD15 may not be LPS, as initially suggested, but muramyl dipeptide (MDP),^{26,27} a component of both Gram negative and positive bacterial cell walls. It has been a consistent and unexplained observation, if somewhat counterintuitive, that both common and rare CD associated variants of the NOD2/CARD15 gene result in reduced NF κ B activity, although these data are from transfection experiments of NOD2/CARD15 gene constructs in embryonic kidney cells.^{27,28} Conversely, the uncontrolled mucosal inflammation of Crohn's disease is characterised by upregulation of NF κ B activation.²⁹

In 2003, Hisamatsu *et al* provided perhaps the most elegant evidence to date, that CARD15/NOD2 may function as an antibacterial factor in CaCo2 intestinal epithelial cells.³⁰ Cells stably transfected with a wild-type CARD15/NOD2 gene construct were able to prevent invasion by *Salmonella typhimurium*. This protective effect was lost in cells transfected with gene constructs of mutant CARD15/NOD2. In the same issue of *Gastroenterology*, Rosenstiel *et al* also demonstrated that NOD2/CARD15 expression in intestinal epithelial cells might be upregulated by the proinflammatory cytokine tumour necrosis factor α (TNF- α).²⁵

Thus with NOD2/CARD15 identification, the emphasis in studies of IBD pathophysiology has shifted to investigations of the innate immune response. The story now develops further with data that suggest that NOD2/CARD15 may be expressed in the Paneth cells of the small intestine³¹, published in this issue of *Gut* [see page 1591]. Ogura *et al* have used the techniques of immunohistochemistry and reverse transcription-polymerase chain reaction to examine NOD2/CARD15 expression in the ileal or colonic tissue from IBD patients and controls. NOD2/CARD15 expression was found to be localised to Paneth cells, within the ileum, or metaplastic Paneth cells within the colon. Indeed, in a parallel paper in *Gastroenterology*, the same authors have extended these observations using *in situ* hybridisation and laser capture microdissection.³² They demonstrated that NOD2/CARD15 expression was enriched in crypts, compared with villi,

and cells expressing NOD2/CARD15 also strongly expressed the proinflammatory cytokine TNF- α , itself a potent stimulus to NOD2/CARD15 expression. However, NOD2/CARD15 expression was not a feature of tissue macrophages in the intestine.

Paneth cells are specialised epithelial cells located mainly in the crypts of the small intestine, in close proximity to epithelial stem cells.³³ Paneth cells secrete antibacterial substances, initially located in granules within the cytosol, in response to prokaryotic rather than eukaryotic pathogens.³⁴ The main antimicrobial factors secreted by the Paneth cell include lysozyme, phospholipase A2, trypsin,³³ α -defensins,³⁴ and angiogenins.³⁵ In the current studies, NOD2/CARD15 expression was noted in close proximity to the secretory granules. Indeed, this close proximity prompts speculation that NOD2/CARD15 may be involved in degranulation and mediator released. It should be borne in mind that Paneth cell degranulation may be triggered not only by MDP but also by other bacterial components.³⁴ One may hypothesise that NOD2/CARD15 might therefore be one of a number of receptors involved in degranulation but functional data would not sustain the hypothesis that it is the sole regulator of this function.

There is increasing interest in the importance of members of the defensin family of molecules in regulating innate immune defences. The α -defensins, which are expressed in Paneth cell granules, are particularly pertinent to the present studies. These cationic cysteine rich peptides are synthesised and stored as precursor proteins,³⁶ and in the mouse release requires lysis of the prodefensin molecule by matrix metalloproteinase 7 (MMP7);³⁷ in humans, this lysis is thought to be mediated by a Paneth cell specific trypsin.³⁸ MMP7 deficient mice have decreased responses to bacterial infections although they do not exhibit chronic intestinal inflammation. It is intriguing to note recent provocative data which suggest that carriage of NOD2/CARD15 variants may be associated with reduced α -defensin release from Paneth cells in response to bacterial cell wall components (J Wehkamp, personal communication, Falk Symposium, Berlin, 2003). Could defective defensin release by the Paneth cell provide the missing link whereby reduced NOD2/CARD15 activity impair host defences to bacteria and underlie persistent intestinal inflammation? The interrelationship between NOD2/CARD15 genotype, NF κ B activity, and Paneth cell secretions clearly bears detailed examination. It is worth mentioning in this context

that the β -defensin 2 gene contains an NF κ B binding site in the promoter region,³⁹ and although this defensin is not a component of Paneth cell granules, it is overexpressed in colonic CD.⁴⁰

In the present study in this issue of *Gut*,³¹ Ogura *et al* were unable to find consistent NOD2/CARD15 expression in colonic mucosa. Only one patient with colonic CD and concomitant Paneth cell metaplasia showed NOD2/CARD15 colonic expression.³² This predominant ileal localisation would explain the association of NOD2/CARD15 mutations with ileal disease. However, conflicting data have recently been published,⁴¹ and the issue remains to be resolved.

The functional role of the Paneth cell, initially identified by Joseph Paneth in Vienna in 1888, has remained unclear for more than 100 years. The recent data ever more strongly implicate the Paneth cell as a contributory factor in the innate immune response to bacteria. It is, of course, of great interest to speculate that this expression is critical in the pathogenesis of chronic CD and further data are eagerly awaited. Of particular interest will be the phenotypic and morphological characteristics of transgenic animals lacking the NOD2/CARD15 gene, and subsequent attempts to reintroduce NOD2/CARD15 protein into the Paneth cells of these animals.

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Aspirin

Aspirin: still learning about the wonder drug

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Aspirin, taken daily over at least one year, may exert chemopreventive effects against the early stages of colorectal carcinogenesis

Preclinical, observational, and clinical data consistently show that non-steroidal anti-inflammatory drugs (NSAIDs)—particularly aspirin—reduce colorectal carcinogenesis.¹ Scores of animal studies show that NSAIDs inhibit the development of colorectal neoplasia across the spectrum of disease, ranging from aberrant crypt foci (ACF) to cancer.² Human data confirm these findings with dozens of observational studies reporting 40–50% reductions in colorectal adenoma incidence, cancer incidence, and cancer associated mortality among aspirin users. The most compelling data were published earlier this year. Two randomised placebo controlled trials conducted in patients at moderate risk for colorectal cancer reported that aspirin administered at doses as low as 81–325 mg/day reduced the development of adenomas by up to 35% after a few years of use.^{3,4} In one trial, greater effects were seen against more advanced lesions.³

Colorectal adenomas are established as common non-obligate precursors of colorectal cancer.⁵ Within the last decade, ACF have been identified in rodent models of carcinogenesis, and have been proposed as precursors of colorectal adenomas and cancers.⁶ With the use of recently developed high resolution/magnifying endoscopes, researchers are now making quantitative (for example, number, size, crypt multiplicity) and qualitative (for example, morphology) real time in vivo assessments of ACF in

humans.⁷ ACF—or at least a subset of them—may represent important risk markers for adenoma-carcinoma development in humans. They may also serve as markers of chemopreventive response. Thus although relatively little is known about ACF and their relevance to more advanced stages of colorectal neoplasia in humans, they provide an important and promising focus for additional research.

In this issue of *Gut*, Shpitz and colleagues⁸ weave together these two investigational threads [see page 1598]. This group describes an association between aspirin use and reduced ACF prevalence and histopathological distribution in ex vivo specimens obtained from 194 patients with colorectal cancer. Among patients who used aspirin for at least one year, they observed a 47% reduction in specimens harbouring ACF, a 64–82% reduction in ACF per cm² of colorectal mucosa, and a 52% reduction in dysplastic ACF. Although ACF reductions were observed in all anatomical sites, the reductions tended to be more dramatic in the distal colorectum. These findings suggest that aspirin taken at 100 mg/day over at least one year may exert chemopreventive effects against the early stages of colorectal carcinogenesis.

While intriguing, these data must be interpreted cautiously. Firstly, the study groups differed with regard to variables that may influence baseline risks for colorectal neoplasia and/or aspirin use, such as gender (males 84% v 52% in the

aspirin and control groups, respectively) and age (aspirin users were much more homogeneous than controls). The investigators do not address the potential impact of these imbalances on the study results, nor did they adjust for them in the analysis. Because we have scant information about ACF, these variables may have confounded interpretations of the effects of aspirin. In addition, the investigators did not report study participants' dietary habits and their use of concomitant medications. Preclinical studies suggest that the latter two exposures may modulate ACF and therefore these limitations may be particularly important.^{9–12} Secondly, without power estimates, it is impossible to know whether the lack of statistical significance for certain variables is true or merely reflects the small sample size. Finally, the selection of the study cohort is not described in detail; therefore, the generalisability of these study results is uncertain.

Despite these limitations, the preliminary findings of Shpitz and colleagues⁸ are stimulating and should prompt additional investigations into whether ACF reductions correlate with or predict aspirin's preventive efficacy against more advanced stages of colorectal neoplasia. These data contribute to a growing body of research suggesting that ACF might be used to identify the preventive efficacy of investigational agents against colorectal carcinogenesis. Takayama *et al* originally reported marked reductions in in vivo colorectal ACF following 12 months of treatment with sulindac.⁷ Both the Shpitz and Takayama studies show that NSAIDs reduce ACF burden after relatively brief exposures. The data of Shpitz and colleagues⁸ add another link to the investigational chain by suggesting that aspirin exerts greater effects against more advanced (that is, dysplastic) ACF. Evidence that aspirin modulates both early and advanced ACF would represent a major advance for the field of chemoprevention research. Needless to say, ultimate validation requires linking NSAID induced reductions in

ACF to reductions in colorectal adenomas, cancer, and/or cancer associated mortality.¹³ Nevertheless, this study moves us closer to a distant but still plausible goal of firmly establishing ACF as meaningful short term markers for cancer prevention research.

The study provides other important insights as well. For example, the effects of aspirin on early colorectal neoplasia appear to be relative, not absolute. While aspirin may have significantly reduced the burden of ACF, all 59 aspirin responsive patients included in the study still developed colorectal cancer. Were ACF reduced to the same extent—or perhaps to a greater extent—among aspirin using patients who did not develop cancer? Do reductions in ACF predict for reductions in adenoma, cancer, or cancer mortality, as suggested by preclinical and observational data? How might we best use or build upon the data generated by Shpitz *et al*? This provocative study leaves a trail of important questions, the answers to which may pave the way for future successes in chemoprevention research.

Shpitz's study⁸ also advances chemoprevention research through its novel investigational design, by using pathological specimens originally obtained to diagnose or treat cancer, allowing for parallel insights into earlier stages of neoplasia. More than 75% of controls had ACF, confirming how common these preinvasive lesions are in patients with colorectal cancer. If this situation is typical, ACF may provide another measure by which the preventive efficacy of agents might be quickly, albeit preliminarily, assessed.

For example, ACF evaluations nested within trials testing drug or dietary interventions—including those intended for other indications—might accelerate

agent development and prioritisation for cancer prevention.¹⁴ This approach could be readily implemented to efficiently test some of the most commonly used drugs or nutritional supplements in Western populations, such as statins, PPAR agonists, NSAIDs, and fibre, all of which may have anticancer properties.^{9–12 15} With the exception of NSAIDs, these have yet to be tested in prospective, randomised, controlled trials. Clearly, observational data derived from ancillary endoscopic assessments in single arm or randomised controlled trials might improve the efficiency and speed of agent identification and testing.

Aspirin has been used in various forms to treat pain and inflammation since as early as 500 BC. The array of indications for aspirin has expanded to include the prevention of myocardial infarction and stroke, clinching its role as the world's first wonder drug.¹⁶ Aspirin now appears to be effective against colorectal neoplasia; why and how we still do not know. New insights into carcinogenesis, such as those provided by Shpitz *et al*, will profoundly alter our expectations for aspirin and its potential to improve the public's health.

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