**Evidence for the involvement of NOD2 in regulating colonic epithelial cell growth and survival**

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_**Abstract**_

**AIM:** To investigate the function of NOD2 in colonic epithelial cells (CEC).

**METHODS:** A combination of _in vivo_ and _in vitro_ analyses of epithelial cell turnover in the presence and absence of a functional NOD2 protein and, in response to enteric _Salmonella typhimurium_ infection, were used. shRNA interference was also used to investigate the consequences of knocking down NOD2 gene expression on the growth and survival of colorectal carcinoma cell lines.

**RESULTS:** In the colonic mucosa the highest levels of NOD2 expression were in proliferating crypt epithelial cells. Muramyl dipeptide (MDP), that is recognized by NOD2, promoted CEC growth _in vitro_. By contrast, the growth of NOD2-deficient CECs was impaired. _In vivo_ CEC proliferation was also reduced and apoptosis increased in _Nod2<sup>-/-</sup>_ mice, which were also evident following enteric _Salmonella_ infection. Furthermore, neutralization of NOD2 mRNA expression in human colorectal carcinoma cells by shRNA interference resulted in decreased survival due to increased levels of apoptosis.

**CONCLUSION:** These findings are consistent with the involvement of NOD2 protein in promoting CEC growth and survival. Defects in proliferation by CECs in cases of CD may contribute to the underlying pathology of disrupted intestinal homeostasis and excessive inflammation.

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**Key words:** Colon; Epithelial cells; NOD2; Growth

**INTRODUCTION**

The intestinal epithelium both acts as a physical barrier and senses and responds to commensal bacteria _via_ expression of pattern recognition receptors (PRRs) that recognize microbe associated molecular patterns (MAMPs). There are two distinct groups of PRRs; the Toll-like receptor family (TLRs) and the NOD-like (nucleotide-binding oligomerisation domain) receptors. The leucine rich repeat sequences of the NOD2 protein are implicated in recognition of fragments of bacterial peptidoglycan (PGN) including muramyl dipeptide (MDP).

NOD2 is expressed in the cytosol of professional antigen presenting cells and epithelial cells exposed to...
microorganisms containing PGN [8]. In cell-based models of NOD2 overexpression, MDP stimulation results in NF-κB activation[3]. This together with the ability of pro-inflammatory cytokines to influence NOD2 expression[8] suggests NOD2 contributes to the innate immune response to microbial pathogens. As intestinal epithelial cells are generally refractory to TLR signals in the absence of inflammation, NOD2 may have additional functions[9]. In the small intestine NOD2 appears to contribute to Peyers patch development[10] and paneth cell production of anti-microbial proteins[11], linking NOD2 and host defense at the epithelial interface.

By contrast, little is known about NOD2 function in the colon. It has been proposed that TLRs control epithelial homeostasis[12]. In considering the cross talk between NOD2 and TLR signaling pathways[13], NOD2 expression in IBD[14] and the central role CARD domain-containing proteins play in regulating apoptosis[15], we determined if activation of NOD2 in CECs is important for promoting CEC turnover and maintaining the integrity of the epithelial barrier. We found that NOD2 contributes to regulating CEC proliferation and survival.

MATERIALS AND METHODS

Animals and infections

Six to nine wk old C57BL/6-Nod2+/+ and C57BL/6-Nod2-/- (F8)[16] mice bred and maintained in the same animal facility were infected by oral gavage with 106 cfu luciferase-expressing Salmonella enterica serovar typhimurium (SL1344-Tn5lux). Biophotonic imaging of all animal mRNA was quantified in freshly isolated CECs cultured for 2 h with MDP (Ac-muramyl-Ala-Disoglutamine) for at least 20 villi from 3 HE-stained sections of colon from 5 mice of each strain prior to and following infection. Axiovision software (Imaging Associates Ltd, Bicester, UK) was used for scaling and measurements.

Flow cytometry

Antibody staining (cytokeratin and CD45) and flow cytometry was used to assess CEC purity. Apoptotic cells were quantified by Annexin V and propidium iodide (PI) or 7AAD staining[17,21]. Levels of caspase 3 activity in cultured CECs were determined using the NucViewTM 488 substrate (Biotium, Hayward, CA) according to the manufacturers' recommendation. Stained cells were analyzed using a FACSCalibur and CellQuest software (BD).

Immunohistochemistry

Paraffin (5 μm) sections were incubated with Ki67 (Dako), caspase 3 (BD-Pharmingen), BrdU (Oxford Biotechnology Ltd, Oxford, UK) or isotype matched control antibodies followed by biotinylated secondary antibodies (Vector Labs) and streptavidin-horseradish peroxidase plus DAB (Vector Labs) or anti-rabbit EnVisionTM labeled polymer (Dako). For BrDU detection sections were pre-treated with 2 mol/L HCl for 30 min followed by neutralization in 0.1 mol/L NaBH4 for 5 min to denature DNA. Stained cells in sections were enumerated using a Zeiss Axiolabor 200 M microscope (Zeiss, Welwyn Garden City, UK) equipped with Axiovision software.

qRT-PCR

NOD2 mRNA was quantified in freshly isolated primary CECs and in HT-29 and SW480 cells using pre-optimized primer sets (Applied Biosystems, Foster City, CA) and an ABI prism 7900HT Sequence Detection System (Applied Biosystems). Threshold cycle (Ct) numbers were determined with Sequence Detection Software (Applied Biosystems) and analysed using the delta Ct comparative method. β-actin was used as a reference gene.

RNaI

NOD2 and scrambled NOD2 shRNA sequences (Dharmacon, Lafayette, CO) were cloned into GFP-expressing lentivector, pNL 3.7. shRNA expressing lentiviruses were prepared using the ViraPower Expression System (Invitrogen). HT-29 and SW480 human colonic carcinoma cells were infected with lentiviruses and 10 mg/mL polybrene (Sigma) for 16 h. Quantitative (Taqman) RT-PCR was used to assess CARD15 mRNA knockdown. Viability and growth of infected (GFP+) and non-infected (GFP-) cells was assessed by flow cytometry as described above.

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We sought NOD2 expression by CECs. The average length in mmol/L and 10 mmol/L EDTA fractions is depicted in the phase contrast photomicrograph insets in phosphatase (AlkP) activity and Ki67 expression, respectively, as described in the Methods section. To determine levels of background staining (dotted open profile). The % value shown represents the frequency of cytokeratin+ cells (%). Cell turnover is altered in NOD2-deficient CECs, although they fully recovered after 3 d. Consistent with NOD2 involvement in epithelial homeostasis, the length of colonic crypts in adult Nod2+/- mice was significantly shorter (143 ± 10 vs 93 ± 5, P < 0.05) compared to that in Nod2+/- mice (Figure 2 and 3A). These differences were also evident after oral infection with a non-lethal dose of Salmonella enterica serovar typhimurium. The average length in Nod2+/- mice was increased upon infection, but was still significantly shorter (134.74 ± 4.22 vs 170.97 ± 6.55, P < 0.05) than in infected Nod2+/- mice at 32 h.

Nod2+/- mice were more susceptible than wild type mice to invasion by Salmonella. During the first 24 h of Salmonella infection the colonic bacterial burden of Nod2+/- mice (cfu at 4 h: 840 ± 161; at 8 h: 1123 ± 187, at 24 h: 631 ± 202) was significantly higher (P < 0.0005) than in Nod2+/- mice (cfu at 4 h: 78 ± 52 88 ± 55; at 24 h: 101 ± 33). Nod2+/- animals also manifested severe diarrhea compared to Nod2+/- mice, which persisted for 36 h, although they fully recovered after 3 d.

CEC turnover in vivo was investigated further by

**Statistical analysis**

All data were assessed for normal distribution using a Shapiro-Wilk test. For parametric and non-parametric data, analysis was performed using Student t-test and Mann Whitney test, respectively using the Student Package for the Social Sciences software (SPSS). P values < 0.05 were considered significant.
BrdU uptake and Ki67 expression. Significantly fewer Ki67+ and BrdU+ CECs were present in Nod2-/- mice compared to Nod2+/+ mice, both prior to and after Salmonella infection (Figures 2, 3B and 3C). Proliferating (BrdU+) epithelial cells also migrated shorter distances from the crypts in Nod2-/- mice compared to Nod2+/+ mice (unpublished observations), consistent with reduced CEC proliferation. Nod2-/- mice also had significantly higher numbers of apoptotic (caspase3+) CECs compared to Nod2+/+ mice after Salmonella infection (1.52 ± 0.37 vs 2.75 ± 0.48, P < 0.001, Figure 3D). These in vivo studies do not, however, exclude the possibility that the effects of NOD2 deficiency on CEC growth and apoptosis are secondary to changes in the activity of mucosal immunocompetent cells.

Growth and apoptosis of Nod2-/- CECs in culture following isolation was analyzed. Primary cultures of Nod2-/- CECs (see Methods section) contained significantly higher (11.5 ± 1.1 vs 17.7 ± 1.4, P < 0.0105) numbers of apoptotic cells compared to Nod2+/+ CECs (Figure 4A) with consistently more cells expressing caspase3 (Figure 4B). The growth of Nod2+/+ CEC in vitro was also significantly less than Nod2+/+ CECs at 24 h (10.7 ± 0.4 vs 8.3 ± 0.2, P < 0.026, Figure 4C). Of note, the addition of MDP to cultures of Nod2+/+ CEC increased cell numbers, whereas no increase in Nod2+/+ CEC number was seen in response to MDP (Figure 4C). The defect in Nod2-/- CEC growth in vitro was correlated with defective MDP-mediated NF-κB activation (Figure 4D).

Neutralization of CARD15 by shRNA reduces human colonic epithelial carcinoma cell survival

Further evidence for the direct involvement of NOD2 in regulating epithelial cell proliferation was sought using RNA interference (RNAi) to knockdown expression of NOD2 in HT-29 and SW480 human colonic carcinoma cells.

After transfection with lentiviral vectors expressing short hairpin RNA (shRNA) NOD2 sequences, the level of CARD15 mRNA in both HT-29 and SW480 cells, as determined by quantitative RT-PCR, was reduced by 75%-80%, compared to both non-treated cells and cells infected with control lentivirus expressing scrambled NOD2 sequences (Figure 5A and data not shown). NOD2 shRNA treatment led to a significant decline in HT-29 cell survival with < 20% of GFP+ (virus infected) cells surviving beyond 6 days (Figure 5B). The decline in GFP+ cells was explained by decreased cell viability and increased apoptosis (Figure 5C). Similar to primary Nod2+/+ and Nod2+/+ CEC, knockdown of NOD2 mRNA had no effect on cell cycle progression, although the number of cycling cells was reduced (unpublished observations) reflecting the increased levels of cell death. By comparison, the growth and survival of HT-29 cells infected with lentiviral vectors expressing scrambled NOD2 shRNA sequences was unaffected (Figure 5B). A similar outcome of NOD2 shRNA on epithelial cell growth was seen in SW480 cells (Figure 5D), although the loss of GFP+ SW480 cells occurred over a longer time period with about 60% reduction in GFP+ cells seen after 4 wk (Figure 5D).
The integrity of the colonic epithelium is maintained by the continual renewal of epithelial cells as a result of the accelerated division of crypt cells that migrate upwards from the base of the crypts. Little is known about the origin and nature of the factors that regulate these processes. Here, we provide evidence for the involvement of NOD2 in regulating murine CEC turnover.

Expression of NOD2 in preparations of CECs enriched for proliferating cells described here, together with prior accounts of increased epithelial expression under conditions of rapid proliferation in vitro and in the inflamed and infected intestine and, the increased severity of chemical-induced colonic mucosal damage in NOD2-deficient mice, suggests NOD2 contributes to regulating epithelial cell turnover and promoting epithelial repair. The low amount of NOD2 expression in the colonic mucosa, under steady-state conditions described here and previously, is consistent with this hypothesis and that altered epithelial cell proliferation and death and increased epithelial permeability contribute to the development of IBD. The absence of intestinal inflammation in Nod2+/- mice, or in mice with a knock-in mutation corresponding to the predominant disease-associated mutant form of human NOD2 in Crohn’s disease, suggests that while NOD2 gene mutations and altered epithelial turnover are a prerequisite for developing Crohn’s disease, they are not sufficient in themselves. Other additional genetic factors or environmental triggers that disrupt the epithelial barrier must also occur for the development of disease.

The absence of NOD2 in CECs does not completely abolish cellular proliferation. NOD2 may, therefore, function as part of a network of interacting and interdependent factors that includes other PRRs and various cytoprotective and repair factors that collectively regulate epithelial homeostasis with other components partially compensating for the absence of NOD2. NOD2 does, however, appear to play a more essential role in promoting the growth and survival of immortalized HT-29 and SW480 colonic carcinoma cells. Differences in the survival curves of these cell lines after NOD2 shRNA treatment may relate to differences in the genetic mutations and their impact on the requirement for NOD2 dependent-survival signals. Additional studies, using primary human CECs and colonic specimens from patients bearing CD-associated
NOD2 mutations, are required to substantiate these findings. However, the inability of HT-29 and SW480 cells to survive NOD2 RNAi treatment is consistent with the disrupted growth of Nod2+/− primary CECs in vitro and in vivo and, that the sustained expression of NOD2 is required to maintain high rates of CEC proliferation. Identifying the pathways and mediators of NOD2 signaling in CECs will help establish how NOD2 contributes to CEC and tumor cell survival.

The proposed role of NOD2 in colonic epithelial homeostasis does not necessarily contradict the notion that it is a bacterial sensor and contributes to innate immunity[27–28]. The importance of NOD2 in innate anti-bacterial responses is demonstrated by the increased bacterial burden and inflammation seen here in Nod2+/− mice after enteric Salmonella infection and previously after infection with Listeria[11], both of which disrupt epithelial barrier function. Cell type specific influences and microenvironmental factors may account for contrasting roles for NOD2 in different studies. The importance of environmental influences is perhaps best demonstrated by its divergent role(s) in the very different environments of the small intestine, where it regulates anti-microbial protein production[13] and GALT development[18], and the colon where it regulates epithelial cell turnover. In addition, differences in the expression of NOD2-regulatory proteins[29,30], or endogenous inhibitors of NOD2[31], could also explain different outcomes of NOD2 activation in different cell types. The recent identification of a NOD2 target gene (DMBT1) that is predominantly expressed in epithelial cells[32] is also consistent with cell type specific NOD2 responses.

How NOD2 carries out its diverse array of functions in different regions of the gastrointestinal tract remains to be determined. In the colon, NOD2’s effect on CEC growth could be direct by, for example, regulating expression of genes and/or proteins involved in cell growth. mRNA profiling of wild type and Nod2−/− CECs by microarray analysis has identified securin (pituitary tumor transforming gene-1) that is required for maintaining appropriate cell division[32] as one of the most underrepresented genes in Nod2−/− CECs (unpublished observation). Alternatively, the involvement of PRRs in the production of cytoprotective and reparative cytokines in the colon[13] and reports of altered patterns of cytokine production by NOD2-deficient cells[18,34] suggest an indirect mechanism of action of NOD2. The growth-promoting effect of NOD2 in CECs contrasts with MDP-induced apoptosis of rabbit kidney epithelial cells, which may reflect cell type specific differences in the response to MDP and its interaction

Figure 4 Decreased survival of Nod2+ CECs in vitro. A: The level of apoptotic cells in cultures of primary CECs from Nod2+/+ and Nod2−/− mice was determined by annexin V and propidium iodide (PI) staining and flow cytometry. Data was collected from 5 experiments and shows the mean (± SE) values. *P < 0.01 B: The frequency of Nod2+ and Nod2− CECs containing caspase3 activity was determined using the flow cytometry based NucViewTM488 Caspase-3 assay. The data represents the mean (± SE) values collected from two experiments. C: The growth of Nod2+ and Nod2− CECs in the absence (Media) or presence of 10 μg/mL muramyl dipeptide (MDP) was determined by comparing the number of viable CEC at the initiation of culture and after 24 h and 48 h. The data shown represents the mean (± SE) values collected from two experiments. D: BF-kB activation in Nod2+/+ (filled bars) and Nod2− (open bars) CEC after exposure to muramyl dipeptide (MDP) was determined by quantitating NF-κB p65 levels in nuclear extracts using a Transfactor Kit as described in Methods section. Values were normalized to control values of cells grown in media alone. The data shown represents the mean (± SE) values from 3 independent experiments.
with different cellular proteins including NOD2. Further studies aimed at identifying NOD2 signaling pathways in CECs will be important in determining how this protein functions in intestinal epithelial cells.

In summary, we have shown that NOD2 contributes to maintaining epithelial cell homeostasis in the colon. Compromised barrier repair may, therefore, underlie aspects of Crohn’s disease where mutant NOD2 alleles contribute to disease.

Peers review
This interesting study was to determine the molecular mechanisms regulating NOD2 function in colonic epithelial cells. The authors conducted both in vivo and in vitro studies to show the specific functions of the NOD2 protein. This is an outstanding and clearly written paper that provides strong evidence for a role for NOD2 in murine colonic epithelial survival.

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