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Rage signalling promotes intestinal tumourigenesis

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Abstract

Development of colon cancer is a multistep process that is regulated by intrinsic and extrinsic cellular signals. Extrinsic factors include molecular patterns that are derived from either pathogens (PAMPs) or cellular damage (DAMPs). These molecules can promote tumourigenesis by activation of the innate immune system, but the individual contribution of ligands and their receptors remains elusive. The receptor for advanced glycation endproducts (Rage) is a pattern recognition receptor that binds multiple ligands derived from a damaged cell environment such as Hmgb1 and S100 protein. Here we show that Rage signalling plays a critical role in sporadic development of intestinal adenomas, since ApccMin/+ Rage−/− mice are protected against tumourigenesis.

Introduction

In past decades, multiple oncogenes and tumour suppressor genes have been identified in colorectal cancer, such as APC, K-Ras and P53. Such genes generally play a role as intrinsic regulators of cancer cell fate, i.e. they affect the behaviour of the mutant cell in a cell autonomous fashion. In more recent years, the immune system has increasingly been recognised as an important extrinsic modifier of tumourigenesis that acts non-cell autonomously (1;2). The link between intestinal inflammation and colon cancer risk is well established. Patients with inflammatory bowel disease are at increased risk of colon cancer development and the risk correlates with both the duration and extent of disease (3). Moreover, only those patients with Crohn’s disease that have colonic involvement of the disease are at increased risk for colon cancer, demonstrating a direct link between inflammation and cancer (4).

The azoxymethane (AOM)-dextrane sulphate sodium (DSS) mouse model of colitis associated colon cancer is a well established mouse model of cancer development in the context of chronic inflammation (5). Using this model it has been shown that activation of pattern recognition receptors such as Toll-like receptor 4 (Tlr4) and the receptor for advanced glycation endproducts (Rage) plays a key role in colitis associated cancer development (6;7). Thus, both pathogen associated molecular patterns (PAMPs), such as bacterial lipopolysaccharide which activates Tlr4, and damage associated molecular patterns (DAMPs) which activate Rage may promote colitis associated tumourigenesis.

Most PAMPs activate the innate immune system through members of the TLR family. Downstream activation comprises of induction of MAP kinase signaling pathways and transcriptional activation of NFκB (8). DAMPs are the endogenous counterparts of PAMPs and downstream signalling activates MAP kinases and NFκB as well. DAMPs can either be secreted by various cell types, or leak from damaged cells. Thereby, DAMPs signal tissue damage to the immune system (9). One of the receptors for DAMPs is Rage, a multiligand receptor that is activated by molecules such as HMGB1, S100 protein, amyloid-β-protein and phosphatidyl serine (10-12).
Thus, in an inflammatory milieu such as colitis, both pathogens and danger signals activate the innate immune system. This promotes carcinogenesis through acute production of cytokines and stimulation of growth, cellular survival and angiogenesis (13). However, a more surprising role for innate immunity in intestinal tumourigenesis was revealed in the absence of overt inflammation in the Apcmin/+ mouse model. These animals have a mutation in the murine homologue of the adenomatous polyposis coli (APC) gene which is frequently mutated in sporadically occurring human colorectal cancers (14). When Apcmin/+ mice were crossed to mice lacking the TLR adaptor protein MyD88, rendering these mice insensitive to TLR activation, adenoma development was virtually completely prevented (2). In later experiments with bone marrow chimeras it was shown that the oncogenic effect of MyD88 was dependent on MyD88-signalling in intestinal epithelial cells and not in bone marrow derived cells (15). Whereas it can be concluded that innate immune activation is an important process in development of sporadic intestinal tumours, little is known about contribution of individual receptors and their ligands to this process. We reasoned that adenomas and colon cancers often harbour necrosis and areas of tissue damage, causing a higher level of DAMPs in these tumours (16-18). Since Rage is activated by DAMPs, Rage may be one of the key receptors that can activate the innate immune system in sporadic adenoma development. We set out to identify a role for Rage signalling in the Apcmin/+ model of sporadic intestinal tumourigenesis.

Results and discussion

We analysed expression of Rage in the normal intestine and in intestinal adenomas using both in situ hybridization (ISH) and immunohistochemistry (IHC). For ISH, three individual probes were generated that were directed against different parts of the Rage coding sequence or against the UTR, as previously described (19). All probes hybridized similarly. Rage mRNA was expressed throughout the epithelium and mesenchyme of the small intestine (Figure 1B). The level of mRNA was high in the crypt and decreased gradually over the length of the villus. To confirm these results we made use of the RageeGFP knockin allele (20). In this mouse, the Rage allele is disrupted by insertion of an eGFP molecule. A functional null allele for Rage remains and eGFP expression reflects Rage promoter activity. We refer to the eGFP knockin allele as Ragee- allele. Staining for eGFP in intestines of Ragee- mice resembled the pattern obtained by ISH (Figure 1A). In the crypts, staining was excluded from Paneth cells. These cells can be distinguished by a pyramidal shape and a content of large secretory granules in the cytoplasm. Of note, crypt base columnar cells, that were identified as intestinal epithelial stem cells (21), were positive for eGFP.

To analyse expression of Rage in intestinal adenomas, we crossed Ragee+/- reporter mice into ApcMin/+ mice. Tumours in ApcMin/+ mice resemble human sporadic adenomas histologically and molecularly. ApcMin/+ Ragee+/- adenomas exibited high expression of eGFP in both epithelium
and mesenchyme. Together these results show that Rage is expressed in the epithelium and mesenchyme of normal intestinal tissue and of mouse intestinal adenomas.

In order to investigate a functional role for Rage signalling in the development of sporadic tumourigenesis, we crossed $Apc^{Min/+}$ $Rage^{+/+}$ mice to homozygosity for the $Rage$-null allele. As controls we used mice from the same crosses that had retained both wild type alleles for $Rage$ (further referred to as $Apc^{Min/+}$ $Rage^{+/+}$ mice). In our hands, $Apc^{Min/+}$ $Rage^{+/+}$ mice developed a polyp number of $111.3 \pm 51.64$ per mouse. This is similar to what has been reported previously.

**Figure 1.** Rage is expressed in epithelium and mesenchyme of the normal intestine. (A) IHC for eGFP reporter molecule showing Rage promoter activity, using an anti-eGFP antibody (Rockland, Goat Polyclonal, 600-101-215). (B) ISH for Rage. (C) IHC for eGFP shows high expression in an intestinal adenoma of an $Apc^{Min/+}$ mouse. (D) IHC for eGFP on a $Rage^{+/+}$ $Apc^{Min/+}$ adenoma as a negative control.
Rage signalling promotes intestinal tumourigenesis in Apc\textsuperscript{Min/+} mice. In contrast, Apc\textsuperscript{Min/+} Rage\textsuperscript{-/-} mice had an average of 46.86 ± 27.58 polyps per mouse, a 58% reduction in tumor number (P < 0.05, Figure 2A). Additional characteristics of tumours, such as size distribution and localisation throughout the intestine and colon were not altered in Apc\textsuperscript{Min/+} Rage\textsuperscript{-/-} mice compared to Apc\textsuperscript{Min/+} Rage\textsuperscript{+/+} (Figure 2B,C). Next we analysed if a reduction in polyp number in Apc\textsuperscript{Min/+} Rage\textsuperscript{-/-} animals could be the result of reduced proliferative capacity of the

Figure 2. Rage signalling promotes tumourigenesis in Apc\textsuperscript{Min/+} mice. (A) Polyp number of Apc\textsuperscript{Min/+} Rage\textsuperscript{-/-} mice and control animals at 17 weeks of age. Each dot represents one animal. (B) Localisation of tumours throughout the intestine. (C) Tumour size distribution. (D) Number of cleaved caspase 3\textsuperscript{+} cells in polyps of Apc\textsuperscript{Min/+} Rage\textsuperscript{-/-} mice and control animals, stained with anti-cleaved caspase 3 antibody (Cell Signalling, Rabbit polyclonal, 9661). Statistical analysis: data are represented as mean ± s.e.m. (A,D) student’s t-tests, (B,C), 2-way ANOVA followed by Bonferroni’s post test for multiple comparisons (** P ≤ 0.01, *** P ≤ 0.001).
intestinal epithelium. We therefore counted BrdU incorporation in non-adenomatous crypts of Apc\textsuperscript{Min/+} Rage\textsuperscript{-/-} mice and controls. However, no changes were observed in BrdU incorporation in Apc\textsuperscript{Min/+} Rage\textsuperscript{-/-} mice (Supplementary Figure 1A). In adenomas Ki67 was used as a marker for proliferation. We assessed epithelial proliferation in size matched adenomas of Apc\textsuperscript{Min/+} Rage\textsuperscript{-/-} mice and control animals. Whereas proliferation seemed to be slightly decreased in adenomas of Apc\textsuperscript{Min/+} Rage\textsuperscript{-/-} mice, the difference was not significant (Supplementary Figure 1B). Possibly, the protection from polyps in Apc\textsuperscript{Min/+} Rage\textsuperscript{-/-} mice resulted from increased cell death, similar to what has previously been described in MyD88\textsuperscript{-/-} Apc\textsuperscript{Min/+} mice. Indeed, the number of cleaved caspase 3\textsuperscript{+} cells was increased in the absence of Rage signalling (Figure 2D). These results show that Rage signalling promotes development of intestinal polyps. A diminished sensitivity to apoptotic epithelial cell death may be one of the factors that contribute to the tumour promoting effect of Rage signalling. To identify those signals that convey survival to tumour cells through Rage, we performed quantitative RT-PCR for Rage ligands on normal tissue and polyps of Apc\textsuperscript{Min/+} Rage\textsuperscript{-/-} animals and controls. In control mice, we found significant upregulation of Hmgb1 and S100A8 but not S100A9 in polyps compared to normal tissue Figure 3). Interestingly, in Apc\textsuperscript{Min/+} Rage\textsuperscript{-/-} mice, Hmgb1 and S100A8 upregulation was not found in polyps. We therefore concluded

![Figure 3. Upregulation of Rage ligands in polyps.](image)

Quantitative RT-PCR for Rage ligands on multiple normal tissue and polyp samples of Apc\textsuperscript{Min/+} Rage\textsuperscript{-/-} (N = 4) and control animals (N = 3). Data are represented as mean ± s.e.m. and significance is calculated with 2-way ANOVA followed by Bonferroni's post test for multiple comparisons (* P ≤ 0.05, *** P ≤ 0.001).
Figure 4. Rage deficiency causes infiltration of mast cells in polyps. (A) Tumor infiltrating immune cells in Rage<sup>−/−</sup> (N = 9) and control mice (N = 7), neutrophils are detected with anti-Ly6 (BD pharmingen 553127, Rat monoclonal), macrophages are detected with anti-F4-80 (BMA biomedicals T2006, Rat monoclonal) T-cells are detected with anti-Cd-3 (Dako cytomation A0432, Rabbit polyclonal) and granulocytes, most notably mast cells are detected with chloroacetate esterase (Leder) staining (Sigma, 90C2). Data are represented as mean ± s.e.m. and significance is calculated with 2-way ANOVA followed by Bonferroni’s post test for multiple comparisons (** P ≤ 0.001). (B) Leder staining on a polyp of a Rage<sup>−/−</sup> and control mouse, arrowhead points at a mast cell.
that Rage signalling activates a positive feedback loop which stimulates the upregulation of its own ligands.

In skin cancer, pro-tumourigenic effects of Rage signalling depend on influx of immune cells into a tumour, which is abrogated in \( \text{Rage}^{+/} \) animals. Therefore, we analysed whether Rage signalling alters immune cell infiltration in intestinal polyps of \( \text{Apc}^{\text{Min}}/\text{Rage}^{-/-} \) mice. Whereas distribution of neutrophils, macrophages and lymphocytes was similar in polyps of \( \text{Apc}^{\text{Min}}/\text{Rage}^{-/-} \) and control animals, we found a surprising fourteen fold increase in cells that stained positive for chloroacetate esterase (Leder stain) in tumours of \( \text{Apc}^{\text{Min}}/\text{Rage}^{-/-} \) mice compared to controls (Figure 4A,B). Leder staining predominantly stains mast cells, but may also react with neutrophils and eosinophils. These results show that in intestinal tumourigenesis lack of Rage signalling causes influx of Leder+ mast cells, although further immune cell distribution is unaltered.

Our current study establishes a role for Rage signalling in the development of sporadic intestinal tumours. Rage is present in both epithelial and mesenchymal cells and expression is increased in intestinal adenomas. \( \text{Apc}^{\text{Min}}/\text{Rage}^{-/-} \) mice that lack Rage, have a 58% reduction in tumour number. The protection of Rage mutant mice to adenoma development may be related to an increased sensitivity to apoptotic cell death in Rage mutant adenomatous cells. Analysis of polyps of \( \text{Apc}^{\text{Min}}/\text{Rage}^{-/-} \) mice shows that transcripts of ligands that activate Rage are upregulated, which is not the case in polyps of \( \text{Apc}^{\text{Min}}/\text{Rage}^{-/-} \) animals. Furthermore, lack of Rage in polyps caused a marked increase of infiltrating Leder+ mast cells that possibly contribute to low polyp numbers.

Whereas previous studies revealed a role for Rage signalling in colitis associated cancer, our current data suggest that Rage signalling also contributes substantially to development of sporadic intestinal tumours. The fact that \( \text{Apc}^{\text{Min}}/\text{Rage}^{-/-} \) mice exhibit protection from tumour formation as well as increased rates of apoptosis are indicative of a pro-survival function for Rage ligands in intestinal tumorigenesis. A similar phenotype was observed in \( \text{Apc}^{\text{Min}}/\text{MyD88}^{-/-} \) mice, (2;15), suggesting that Rage and MyD88 signalling share tumour promoting mechanisms. Although it was long known that both pathways activate similar downstream effectors, it was only recently suggested that Rage signalling may require MyD88 as an adaptor protein (23). The oncogenic effect of MyD88 in adenoma formation may therefore be explained, in part, by Rage signalling. Since the protection from adenoma formation was more profound in MyD88 mutant mice than in Rage knockout mice, other TLRs and their ligands likely play an additional role in spontaneous tumourigenesis. Our finding that Rage-ligands such as \( \text{Hmgb1} \) and \( \text{S100a8} \) are upregulated in polyps corroborates findings in humans, where these ligands are upregulated in colorectal carcinomas (7;17). We found that upregulation of Rage ligands in polyps depended on Rage signalling. This was also observed previously in DMBA/TPA induced skin carcinogenesis (24). This confirms the existence of a positive feedback loop which induces transcription of Rage ligands upon activation of Rage signalling. In the work by Gebhardt et al. however, the protective phenotype of \( \text{Rage}^{-/-} \) mice was shown to depend on decreased influx of immune cells, whereas we find no evidence of reduced immune cell influx during intestinal adenoma formation. Instead
we find a surprising extensive infiltration by Leder+ cells in adenomas of ApcMin/+ Rage−/− mice. Although Leder staining may react with other types of immune cells such as neutrophils and eosinophils, it predominantly stains mast cells. In animal models of intestinal tumorigenesis, both anti- and protumorigenic roles of mast cells have been observed (25). It is therefore unclear whether the extensive infiltration of mast cells or potentially other types of Leder+ cells may have contributed to reduction in the polyp numbers in ApcMin/+ Rage−/− mice.

A picture emerges where upon tissue damage within intestinal polyps, increased amounts of Rage ligands are made. Signalling through Rage sustains high levels of these ligands and upholds a tissue damage response that emits pro-survival signals protecting cells from apoptosis. In mice that lack Rage, the cycle of inflammation is blocked, resulting in increased apoptosis and a decline in tumor number.

Identifying triggers that promote cancer leads to better understanding of this disease and may open doors to therapeutic options. Tumours often contain cellular damage and DAMP-signalling is important for tumour progression (26). We identify Rage as a receptor that may transduce these signals to promote tumorigenesis. Targeting Rage may therefore be of interest in the prevention and treatment of colon cancer.

**Methods**

**Animal experiments**

All experiments were performed according to the Leiden University Medical Center animal experimental committee guidelines.

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Reference List


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Figure 1. Proliferation is not altered in Apc$^{Min}$ Rage<sup>−/−</sup> mice.
(A) Apc$^{Min}$ Rage<sup>−/−</sup> animals and controls were injected with 200 µl BrdU (10 mg/ml in PBS). Sections were stained with anti-BrdU antibody (Roche, mouse monoclonal, clone BMC9318). Blinded to treatment group, BrdU<sup>+</sup> cells per crypt were counted in at least 30 crypts per animal of Apc$^{Min}$ Rage<sup>−/−</sup> animals versus Apc$^{Min}$ Rage<sup>+/+</sup> controls (n=10 per group). (B) Ki67<sup>+</sup> cells in size matched adenomas of Apc$^{Min}$ Rage<sup>−/−</sup> animals versus Apc$^{Min}$ Rage<sup>+/+</sup> controls. IHC was performed using an anti-Ki67 antibody (Monosan, mouse monoclonal, Clone MM1). Data are presented as mean ± s.e.m. and analysed with student’s t-tests.