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Intestinal inflammatory cytokine response in relation to tumorigenesis in the *Apc*$^{Min/+}$ mouse

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ABSTRACT

The etiology of colon cancer is a complex phenomenon that involves both genetic and environmental factors. However, only about 20% have a familial basis with the largest fraction being attributed to environmental causes that can lead to chronic inflammation. While the link between inflammation and colon cancer is well established, the temporal sequence of the inflammatory response in relation to tumorigenesis has not been characterized. We examined the timing and magnitude of the intestinal inflammatory cytokine response in relation to tumorigenesis in the *Apc*$^{Min/+}$ mouse. *Apc*$^{Min/+}$ mice and wildtype mice were sacrificed at one of 4 time-points: 8, 12, 16, and 20 weeks of age. Intestinal tissue was analyzed for polyp burden (sections 1, 4 and 5) and mRNA expression and protein concentration of MCP-1, IL-1β, IL-6 and TNF-α (sections 2 and 3). The results show that polyp burden was increased at 12, 16 and 20 weeks compared to 8 weeks ($P < 0.05$). Gene expression (mRNA) of MCP-1, IL-1β, IL-6 and TNF-α was increased in sections 2 and 3 starting at week 12 ($P < 0.05$), with further increases in MCP-1, IL-1β and IL-6 at 16 weeks ($P < 0.05$). Protein concentration for these cytokines followed a similar pattern in section 3. Similarly, circulating MCP-1 was increased at 12 weeks ($P < 0.05$) and then again at 20 weeks ($P < 0.05$). In general, overall polyp number and abundance of large polyps were significantly correlated with the inflammatory cytokine response providing further support for a relationship between polyp progression and these markers. These data confirm the association between intestinal cytokines and tumorigenesis in the *Apc*$^{Min/+}$ mouse and provide new information on the timing and magnitude of this response in relation to polyp development. These findings may lead to the development of inflammatory mediators as important biomarkers for colon cancer progression. Further, these data may be relevant in the design of future investigations of therapeutic interventions to effectively target inflammatory processes in rodent models.

1. Introduction

Colon cancer is a significant global health concern; despite advances in detection, surgery and chemopreventive treatment it remains the third most common malignancy and the fourth most common cause of cancer mortality worldwide [1–3]. The etiology of colon cancer is a complex phenomenon that involves contribution from both genetic and environmental factors. However, only about 20% of colon cancer cases can be attributed to genetic factors [4] with the vast majority of cases being ascribed to environmental causes that can lead to chronic inflammation. For example, inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's colitis, all of which display chronic inflammation of the gastrointestinal mucosa, are associated with increased risk for the development of colon cancer [5,6].

The link between inflammation and colon cancer is well established; inflammation has been linked to every step involved in the development and progression of colon cancer, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis [7–11]. Furthermore, chronic inflammation has been associated with "sickness behaviors" including circadian disruptions, anorexia, cachexia, fatigue, and decreased physical activity all of which can lead to a decreased quality of life, as well as poorer prognosis and survival in cancer patients [12–14].

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The inflammatory cytokines interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor-α (TNF-α), largely produced by macrophages, have all been associated with poor outcome in colon cancer [7–10]. In fact, we have previously shown that IL-6 overexpression using electron gene transfer techniques can increase polyp burden in the $Apc^{Min/+}$ mouse model of intestinal tumorigenesis [15]. Similarly monocyte chemoattractant protein 1 (MCP-1), a major chemokine for macrophage recruitment, has been associated with increased grade of the tumor in certain cancers [16,17]. While the link between inflammatory cytokines and colon cancer is well recognized, the timing and magnitude of this response in relation to tumorigenesis has not been described.

The $Apc^{Min/+}$ mouse model has been the most widely used genetically engineered mouse model for cancer studies that involve the gastro-intestinal tract [18,19]. It was the first mouse model to be generated by mutation of the ademonatous polyposis coli (Apc) gene through random chemical carcinogenesis [20]. This gene is similarly mutated in patients with familial adenomatous polyposis [21]. It has been shown to be responsive to treatment with anti-inflammatory agents, including both anti-inflammatory dietary supplements as well as non-steroidal anti-inflammatory drugs (NSAIDs) [18,19]. Despite this, intestinal inflammation has not been characterized in this model; to our knowledge there are no studies that have examined the relationship between inflammatory cytokines and tumor burden in the $Apc^{Min/+}$ mouse.

The purpose of this study was to examine the temporal sequence and magnitude of the inflammatory cytokine response in relation to tumorigenesis in the $Apc^{Min/+}$ mouse. This may lead to the development of inflammatory mediators as important biomarkers to assess progression of this disease, and further, allow for the determination of appropriate timing of effective treatments to target inflammatory processes in mouse models.

2. Materials and methods

2.1. Animals

$Apc^{Min/+}$ male mice on a C57BL/6 background (Jackson Laboratories) were purchased and bred with female C57BL/6 mice in the University of South Carolina's Center for Colon Cancer Research (CCCR). Offspring were genotyped as heterozygotes by RT-PCR for the *Apc* gene by taking tail snips at weaning. The primer sequences were sense: 5′-TGAGAAAGACAGAAGTTA-3′; and antisense: 5′-TTCCACTTTGGCATAAGGC-3′. Female $Apc^{Min/+}$ offspring were randomly assigned to one of four different timepoints: 8, 12, 16 or 20 weeks of age (n = 8–12/group). Wildtype C57BL/6 mice were used as age matched controls (n = 6–11/group). Mice were maintained on a 12:12 h light–dark cycle in a low-stress environment (22 °C, 50% humidity and low noise) and provided food and water ad libitum. All animal experimentation was approved by the University of South Carolina's Institutional Animal Care and Use Committee.

2.2. Tissue collection

Mice were sacrificed at their respective group age (8, 12, 16 or 20 weeks) for tissue collection using isoflurane overdose. All mice were sacrificed in the mornings between 9:00 and 11:00am. The small intestine was carefully dissected distally to the stomach and proximal to the cecum. The large intestine (section 5) was removed from the distal end of the cecum to the anus. Mesentery tissue was removed with tweezers, and the small intestine was cut into four equal sections (sections 1–4). All intestinal sections were flushed with PBS, opened longitudinally, and flattened with a cotton swab. Sections 1 and 4 of the small intestine and the large intestine (section 5) were fixed in 10% buffered formalin (Fisher Scientific, Pittsburg, PA) for 24 h. Sections 2 and 3 were divided into two equal parts and mucosal scrapings were performed in iscoves medium (Invitrogen, Carlsbad, CA) (containing 5% fetal bovine serum and a cocktail enzyme inhibitor (10 mM EDTA, 5 mM benzamidine HCl, and 0.2 mM phenylmethyl sulfonyl fluoride)) and TRIzol reagent (Invitrogen, Carlsbad, CA) for protein and gene expression analysis, respectively. Samples were stored at −80 °C until analysis of inflammatory mediators. Blood was collected from the inferior vena cava using a heparinized syringe and spun in a microcentrifuge at 4,000 rpm for 15 min. Plasma was then stored at −80°C until assayed for MCP-1.

2.3. Polyp counts

Formalin-fixed intestinal sections from all animals were rinsed in deionized water, briefly stained in 0.1% methylene blue, and counted by the same investigator who was blinded to the treatments. Polyps were counted under a dissecting microscope, using tweezers to pick through the intestinal villi and identify polyps. Polyps were categorized by size (>2 mm, 1–2 mm, and <1 mm).

2.4. Expression of inflammatory markers

Procedures for RNA isolation from mucosal scrapings were performed as previously described [22,23]. Briefly, mucosal tissue was homogenized under liquid nitrogen with a polytron, and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). The extracted RNA (2.5 μL of sample) was dissolved in DEPC-treated water and quantified spectrophotometrically at 260-nm wavelength. RNA quality was assessed on an Agylent 2100 BioAnalyzer. RNA was reverse transcribed into cDNA in a 50 μL reaction volume containing 19.25 μL RNA (1.5 μg) in RNase-free water, 5 μL 10× RT Buffer, 11 μL 25 mM $MgCl_2$, 10 μL deoxyNTPs mixture, 2.5 μL random hexamers, 1 μL RNase inhibitor, and 1.25 μL multiscribe reverse transcriptase (50 U/μL). Reverse transcription was performed at 25 °C for 10 min, 37 °C for 60 min, and 95 °C for 5 min, followed by quick chilling on ice and storage at −20 °C until subsequent amplification. Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) analysis was done per manufacturer's instructions (Applied Biosystems, Foster City, CA) using TaqMan® Gene Expression Assays (IL-1β, IL-6, TNF-α and MCP-1). DNA amplification was carried out in 12.5 μL Taqman Universal PCR Master Mix (AmpliTaq Gold DNA Polymerase, Passive Reference 1, Buffer, dNTPs, AmpErase UNG), 1 μL cDNA, 9 μL RNase-free water, and 1.25 μL 18S primer (VIC) and 1.25 μL primer (FAM) (for endogenous reference and target gene) in a final volume of 25 μL/well. Samples were loaded in a MicroAmp 96-well reaction plate. Plates were run using Applied Biosystems Sequence Detection System. After 2 min at 50 °C and 10 min at 95 °C, samples were coamplified by 40 repeated cycles of which one cycle consisted of 15 s denaturing step at 95 °C and 1 min annealing/extending step at 60 °C. Data were analyzed using Applied Biosystems software using the CT, cycle threshold, which is the value calculated and based on the time (measured by PCR cycle number) at which the reporter fluorescent emission increases beyond a threshold level (based on the background fluorescence of the system), and it reflects the cycle number at which the cDNA amplification is first detected. All samples were run in duplicate.

Quantification of cytokine gene expression for IL-1β, IL-6, TNF-α and MCP-1 were calculated using the delta CT method as described by Livak and Schmittgen (2001) [24]. Briefly, delta CT (CT(FAM) - CT(VIC)) is calculated for each sample and control. Delta delta CT (delta CT(control) – delta CT(sample)) is then calculated for each sample and relative quantification is calculated as 2 delta delta CT. Initial exclusion criteria consist of FAM CT $\geqslant$ 40 and VIC