Chronic Inflammation Promotes Retinoblastoma Protein Hyperphosphorylation and E2F1 Activation

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Abstract

Chronic inflammation contributes to tumorigenesis. The retinoblastoma protein (pRb), in its hyperphosphorylated form, releases E2 promoter binding factor-1 (E2F1), which drives cell proliferation. Here, we show that pRb is hyperphosphorylated in both mouse and human colitis. In turn, pRb hyperphosphorylation is associated with release of E2F1 from pRb, resulting in the activation of E2F1 target molecules involved in proliferation and apoptosis. These observations provide insight into the in vivo mechanisms associated with chronic colon inflammation and increased colon cancer risk.

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Introduction

Chronic inflammation is procarcinogenic (1). Ulcerative colitis is a chronic inflammatory disease of the colon and people who have the disease have an increased colon cancer risk (2). Although the mechanisms involved in the increased risk are unclear, ulcerative colitis is associated with increased colonic epithelial cell proliferation (3–5). The driving forces of the proliferation, however, remain unresolved.

During acute inflammation, activated lymphocytes and leukocytes release necessary cytokines, prostaglandins, and free radicals to resolve tissue injury and clear microbial pathogens. However, in states of chronic inflammation, constitutive cellular activation and release of proinflammatory factors can damage otherwise healthy neighboring epithelial cells thus driving carcinogenesis by altering targets and pathways crucial to normal tissue homeostasis (1).

The retinoblastoma protein (pRb) phosphoprotein is a key player in the regulation of cell growth and proliferation. Under most cellular conditions, pRb controls cell cycle entry by sequestering the E2F family of transcription factors. Upon receiving appropriate growth signals, pRb is hyperphosphorylated by cyclin-dependent kinase (cdk) complexes such as cyclin D/cdk4/6 and cyclin E/cdk2 (6). The pRb hyperphosphorylation results in E2F release and transcription of growth-associated genes.

Based on pRb’s direct ability to regulate the cell cycle through phosphorylation status, and that pRb phosphorylation is altered following exposure to free radicals and other inflammatory species (7–10), we hypothesized that pRb phosphorylation status and its pathway are dysregulated in ulcerative colitis in vivo. We show here that results are consistent with this hypothesis.

Materials and Methods

Mouse model of colitis. Seven-week-old C57BL/6 mice received either water ad libitum, or 3% dextran sodium sulfate (DSS) for three cycles. Each cycle in the DSS group consisted of 3% DSS in drinking water for 7 days followed by a 7-day interval with normal drinking water. The nontreated group was given normal drinking water for the duration of the experiment. Following completion of the third cycle, mice were euthanized and colonic tissue was obtained. Colon tissue samples were washed with PBS and formalin fixed and paraffin embedded for pathology and immunohistochemistry. Colonic epithelial cells were obtained from scrapings of full-length colons and frozen immediately at −80°C.

Human colitis tissues. Noncancerous, ulcerative colitis colon tissues were obtained from the Cooperative Human Tissue Network (Philadelphia, PA). The study was approved by the Institutional Review Boards of the University of South Carolina, and the NIH. Twenty-five archived colonic surgical tissue samples from 25 patients with ulcerative colitis were available for analysis.

Immunoprecipitation and Western analysis. For immunoprecipitation and Western blot analysis, whole colon epithelial cell lysates were obtained as described previously (11). Part of the lysate was saved for Western blot analysis of pRb phospho-Ser780 (antibody: Cell Signaling, Beverly, MA; 9307), E2F1 (antibody: BD PharMingen, San Diego, CA; 554213), inducible nitric oxide synthase (iNOS; antibody: Cayman, Ann Arbor, MI; 160862-1EA), p53 (CM-1; Signet Laboratories, Dedham, MA), p19ARF (Abcam, Cambridge, MA; Ab 80), proliferating cell nuclear antigen (PCNA; antibody: Abcam, ab2426), phospho-Akt-Ser173 (Cell Signaling, 9271), cyclin D1 (EMD Biosciences, San Diego, CA; Ab-3), and actin (EMD Biosciences, Ab-1). The remaining lysate was used for immunoprecipitation of pRb to evaluate E2F1 interactions. Whole cell extracts (400 μg) were incubated with agarose-conjugated mouse monoclonal anti-pRb antibody (EMD Biosciences, Ab-1) while rotating for 2 hours at 4°C. After centrifugation (5,000 rpm, 1 minute), fresh agarose-Ab complex was again added to the supernatant and incubated 1 hour at 4°C. The pellet (agarose-Ab complex) was then washed five times with cold lysis buffer, and immunoprecipitated protein was extracted from agarose-Ab complex with Laemmli buffer and heat denaturation. Immunoprecipitated protein was separated by SDS-PAGE, electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA), and probed with E2F1 (BD PharMingen) or pRb (Abcam, ab2426) antibodies. No interaction was defined as a complete lack of E2F1 detection following pRb immunoprecipitation, pRb-deficient cells (e.g., Saos-2) and nonspecific IgG were used as the negative controls (data not shown). Cells positive for

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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iNOS (cytokine-stimulated ANA-1 mouse macrophages), pRb/E2F1/p53/p19ARF/PCNA/cyclin D1 (HCT 116), and phosphor-AKT (A20) were used as positive controls. To avoid loss of our entire sample if an experimental error occurred, we did immunoprecipitation experiments on sequential days. Half the samples (water treated and DSS treated) were immunoprecipitated and immunoblotted on 1 day; the others immunoprecipitated and immunoblotted on the other day. These blots, therefore, had to be digitally merged (Fig. 4A). All images were quantified using the Band Leader (version 3.0) software.

**Immunohistochemistry.** Serial sections of both mouse and human colon tissues were incubated with antibodies against pRb (human: monoclonal clone 4H1, Cell Signaling; mouse: rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), or phospho-specific antibodies directed to Ser780 (Cell Signaling), or Ser807/811 (Cell Signaling) of pRb. Following incubation with primary antibody, sections were washed with PBS supplemented with 0.5% Tween 20 (PBST) and incubated with a biotinylated anti-mouse or anti-rabbit IgG, then a horseradish peroxidase–conjugated avidin-biotin complex (DAKO, Carpinteria, CA). The chromogen was 3,3′-diaminobenzidine, and sections were counterstained with 1% methyl green. Immunoabsorbed antibodies were used as negative controls. The positive control tissue was colon cancer sections. These sections (from three different subjects) were highly positive for pRb, pRb phospho-Ser780, and pRb phospho-Ser807/811 (data not shown).

**Quantification of immunohistochemistry.** Scoring of immunostained tissues was facilitated by use of a computer-interfaced morphometric digitizing system. This included a DP70 digital camera and Image-Pro Plus analysis software (Opielco, Dulles, VA). The number of cells per unit area in an H&E section from human or mouse colon was calculated by counting ~ 5,000 cells (mean: human colon, 0.076 cells per unit area; mouse colon, 0.027 cells per unit area). This did not differ between groups. For each immunostained section, the number of positive cells per a minimum of 1,000 cells was calculated. Interindividual and intrindividual differences in scoring were monitored for each assay. Similar quantification techniques have been used previously (12).

**RNase protection assay.** Total RNA was isolated using QuickPrep extraction kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) followed by RNeasy total RNA (Qiagen, Valencia, CA) extraction and dissolved in RNase-free water. Expression of indicated genes were examined with an RNase protection assay using mouse multiprobe RNA probe template sets (BD PharMingen). Riboprobes were synthesized in the presence of [32P]dUTP to yield labeled antisense RNA probes. The RNase protection assay was done on 10 μg of individual total RNA samples using a RiboQuant multiprobe RNA protection assay kit (BD PharMingen). Protected fragments were separated on 5% polyacrylamide denaturing nucleic acid separation gels, dried, and exposed to a phosphor-imaging screen. The intensity of protected bands were quantified using a phosphor-image analyzer. The expression of individual gene transcripts were normalized to the intensity of housekeeping genes L32 and GAPDH.

**Statistical analysis.** Mean differences in marker levels were compared by a Student’s t test. P = 0.05 was considered significant. A Pearson correlation coefficient was applied for comparisons of the trends.

**Results and Discussion**

**pRb is hyperphosphorylated in mouse and human colitis.** pRb hyperphosphorylation is associated with entry into the cell cycle and proliferation (13, 14). Ulcerative colitis patients have elevated cellular proliferation indices in their colons, and this is suspected to play a role in the increased colon cancer risk (3–5). To test whether pRb is hyperphosphorylated in human colitis, we immunostained serial colon sections from 25 individuals with ulcerative colitis and compared them with colon sections from subjects without colitis and patients with colon cancer. Figure 1A shows that patients with colitis have significantly elevated pRb phosphorylation at Ser780 (P < 0.01) and Ser807/811 (P < 0.01). Levels are similar to those observed in patients with colon cancer (data not shown). The 780 and 807/811 phosphorylation sites were chosen because they reside in the area of E2F1/pRb binding and may play a key role in the interaction between these two molecules (15–17). Figure 2 shows representative immunohistochemical staining of serial sections a subject with colitis and a subject without colitis ("normal"). Enhanced pRb and phospho-pRb levels were consistently observed at the base and proliferating zones of crypts in colitis samples, whereas little to no staining was observed in normal colonic epithelium.

To compliment observations made in patients with colitis, we extended our studies to a mouse model of colitis. Compared with the water-fed control group, mice fed 3% DSS for three cycles had significantly elevated colitis (Supplementary Fig. 1). As shown in Fig. 1B, associated with mouse colitis was a significant elevation in pRb phosphorylation at Ser780 (P < 0.01) and Ser807/811 (P < 0.01). Figure 3 shows representative photomicrographs of pRb phosphorylation observed in noninflamed (normal) mouse colons or mice treated with DSS to induce colitis ("colitis").

**E2F1 is released from pRb in colitis.** Previous studies have shown that phosphorylation of Ser780 and Ser807/811 causes E2F1 release and the activation of downstream genes (6, 15–17). This suggests that pRb may be inactivated during inflammation and

**Figure 1.** pRb is hyperphosphorylated in colon tissues from patients with ulcerative colitis (A) and in mouse colitis (B) at Ser780 and Ser807/811. Percentage of cells that were stained with antibodies against indicated pRb isoforms. Numbers under columns are the total number (n) of samples observed for each endpoint. A different but serial set of samples was used for each endpoint. Quantification was done using a computer-based system, as discussed in Materials and Methods. Immunoabsorbed antibodies were used as negative controls. *, P < 0.05 (at Ser780) and **, P < 0.05 (at Ser807/811), significant increases in pRb phosphorylation compared with normal colon.
release E2F1, which in turn, can activate downstream genes that control cell cycle progression and apoptosis. To test this hypothesis, we collected epithelial cells from the colons of control-treated and DSS-treated mice and examined the phosphorylation status of pRb and association with E2F1. Following immunoprecipitation with an anti-pRb antibody, E2F1 was probed to determine whether E2F1 was still attached to pRb in mouse colitis. There was a noticeable lack of coimmunoprecipitation of pRb with E2F1 in inflamed colons (DSS treated) compared with water-treated controls. All samples from the colitis group had none or a reduced pRb/E2F1 interaction compared with the water-treated group (Fig. 4A). To verify that pRb was phosphorylated after induction of colitis, we probed whole cell lysates for pRb phospho-Ser780. As a marker of inflammation, we probed for iNOS and cyclooxygenase-2 (COX-2). Colon tissue lysates from the entire colitis group expressed elevated levels of iNOS (Fig. 4A and B) and COX-2 (data not shown). Lysates from water-treated control samples had little or no iNOS or COX-2 expression, whereas these factors were induced ~25-fold in the DSS-treated mice (Fig. 4B). Interestingly, there was a strong negative correlation between pRb/E2F1 interactions and elevated iNOS expression (correlation coefficient = –0.84), suggesting that inflammation, marked by iNOS, is associated with E2F1 release from pRb.

**E2F1 release correlates with activation of gene targets in colitis.** Increased cell proliferation contributes to the increase in genomic instability observed in patients with colitis (11, 18). Therefore, we examined whether the release of E2F1 is associated with the activation of E2F1 target proteins involved in proliferation. We first probed lysates for PCNA, which is a direct target of E2F1 (19). There was a significant increase in PCNA expression in the colons of DSS-treated mice compared with water-treated mice (P < 0.05; Fig. 4A and B), and this was directly correlated with limited pRb/E2F1 interactions, indicating PCNA is activated in many of the samples showing E2F1 release. We also saw an increase in the levels of cyclin D1, another target of E2F1 (20). Recently, it has been shown that Akt is a target of E2F1 and that activated Akt (phospho-Akt-Ser773) inhibits E2F1-mediated apoptosis (21). We therefore probed our blots with an anti-phospho-Akt-Ser773 antibody and found there to be a significant increase in Akt phosphorylation in mice with colitis (P < 0.05; Fig. 4A and B). Finally, to better understand the pRb pathway kinases activated in colitis, we probed normal colons and mouse colitis using an RNase protection assay. Figure 4C shows an up-regulation of cdk2 and cdk4, indicating that these kinases may be appropriate targets for chemoprevention of colitis-associated colon cancer. The findings of increased cyclin D1 levels are consistent with previous studies showing increased levels in colon adenomas (22).

**Conclusions**

Overall, our results are consistent with the hypothesis that inflammation contributes to pRb hyperphosphorylation and release of E2F1, which in turn, activates genes that are associated with proliferation (PCNA and cyclin D1) and apoptosis inhibition.
Recently, it has been shown that increased E2F1 activity can also lead to genomic instability (23). Based on the increased levels of DNA damage observed in colitis patients (18), our findings are consistent with a novel link associating pRb hyperphosphorylation and genomic damage during colitis. Previously, we have shown that p53 is mutated early in colitis (24), and that DNA damage in colitis can lead to a stress response with accumulation and activation of wild-type p53 through posttranslational modifications (11). Because E2F1 may be a part of a DNA damage stress response pathway (25) through the activation of p53 via the E2F1 target, p19 ARF (26), we tested whether p19ARF and p53 are also activated in our mouse colitis samples. Figure 4A and B shows both are increased in colitis, consistent with the hypothesis that E2F1 release may be a part of an inflammatory stress response pathway in patients with colitis through the activation of p53. This is consistent with a model of pRb and p53 pathways as a complex network rather than two simple linear pathways in colitis. During these early phases of carcinogenesis, cancer development is associated with a stress response involving the pRb and p53 pathways, with the generation of genomic instability and possible selective outgrowth of p53 mutant cells. Our finding here, together with the recent findings that the proinflammatory cytokine, macrophage migration inhibitory factor, interferes with the pRb (27) and p53 pathways (28), provides evidence that both pathways are involved in the association between chronic inflammation and cancer.

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