Overexpressed β-Catenin Blocks Nitric Oxide–Induced Apoptosis in Colonic Cancer Cells

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Abstract

β-Catenin plays an important role in colonic tumorigenesis whereas inducible nitric oxide synthase and nitric oxide are elevated in colonic inflammation. Resistance of colonic epithelial cells to the induction of apoptosis may contribute to tumor development. Nitric oxide can stimulate apoptosis and, paradoxically, is implicated in the development of colon cancer. Our hypothesis was that β-catenin could increase the resistance of colonic cancer cells to nitric oxide–induced apoptotic cell death. Here we show, using a β-catenin overexpression system, that increased cytosolic β-catenin renders colonic epithelial cells more resistant to nitric oxide–induced apoptotic cell death, independently of nitric oxide–induced accumulation of p53. Furthermore, we show that this occurs through inhibition of nitric oxide–induced release of cytochrome c from mitochondria and by blocking both the nitric oxide–induced suppression of the antiapoptotic protein, Bcl-xL, and the phosphorylation of Akt. We contend that increased nitric oxide production, such as that which occurs in chronic colonic inflammation, may select the cells with oncogenic mutant β-catenin regulatory genes and contribute to human colonic carcinogenesis and tumor progression. (Cancer Res 2005; 65(19): 8604-7)

Introduction

Inflammatory bowel disease is associated with an increased risk of colon cancer (1) and is characterized by the induction of several proinflammatory genes including those encoding inducible nitric oxide synthase (2). Nitric oxide is involved in several physiologic and pathophysiologic processes, including apoptosis in macrophages, thymocytes, certain neurons, and tumor cells. However, the role of apoptosis is controversial because it may also inhibit apoptosis in other cells including hepatocytes (3). Inducible nitric oxide synthase is highly expressed by macrophages and epithelial cells during gastrointestinal inflammation and activation of inducible nitric oxide synthase can generate mutagenic concentrations of nitric oxide which may be sustained in the micromolar range for a long period (4). Nitric oxide triggers epithelial apoptosis in a p53-dependent manner (3) and may thus clonally select precancerous cells with mutant p53, which are incapable of undergoing apoptosis (4). This supports the hypothesis that prolonged exposure to nitric oxide in conditions like chronic inflammation may predispose cells to tumorigenesis (4).

In addition to the shown role of p53 in tumorigenesis, defects in the regulation of β-catenin are also associated with many colon cancers (5). β-Catenin is an E-cadherin–associated protein involved in cell-cell adhesion at the level of the adherens junction. It can, in certain circumstances, be released into the cytosol where it is regulated by the adenomatous polyposis coli/axin/glycogen synthase kinase-3β complex through phosphorylation, ubiquitination, and degradation processes (6). However, during Wnt signaling, or in cases where the regulatory complex is mutated or otherwise dysfunctional, such as the Apc mutations characterizing many colon cancers (7), cytosolic concentrations of β-catenin increase, allowing it to translocate to the nucleus where, through its interaction with Tcf, it acts as a transcription factor of genes associated with cell proliferation, such as cyclin D1 (8). Moreover, activation of the Wnt pathway or β-catenin has been shown to promote cell survival (9–12).

Although there is some evidence for the coincidence of activated β-catenin and high expression of inflammatory mediators, such as nitric oxide, little is known about how β-catenin interacts with inducible nitric oxide synthase/nitric oxide to play a role in the transition from inflammation to cancer. In rat colonic epithelium, altered expression of β-catenin is related to the expression of inducible nitric oxide synthase (13). It has been shown in human colonic cells that β-catenin interacts with nuclear factor κB (14), which is also a key modulator of inflammation and inducible nitric oxide synthase expression in intestinal epithelial cells. Activation of β-catenin leads to inhibition of trophoblast apoptosis following increased expression of inducible nitric oxide synthase (15). However, the relationships among nitric oxide, β-catenin, and apoptosis in colon cancer are not known. In this study, we show that overexpression of β-catenin in colonic cells blocks nitric oxide–induced, p53-mediated apoptosis through Bcl-xL– and Akt–related pathways.

Materials and Methods

Cell culture. The human colon cancer cell lines RKO and RKO-E6 from American Type Culture Collection (Manassas, Virginia) were grown in DMEM/F12 (Sigma, Oakville, Ontario). RKO-E6 and transfected cell lines were maintained in complete medium containing 500 μg/mL Geneticin (Life Technologies, Inc., Burlington, Ontario). To make a stable β-catenin–overexpressing transfectant cell line, RKO cells were transfected with a β-catenin S37A expression vector (pcDNA3–β-catenin S37A) or the control vector pcDNA3 (Invitrogen, Burlington, Ontario) using Lipofectamine (Invitrogen) and selected in 500 μg/mL Geneticin.

Flow cytometry. Cells were harvested and fixed in 70% ethanol at −20°C overnight. Cells were then treated with RNase (1 mg/mL), stained with propidium iodide (1 mg/mL), and analyzed using a Becton Dickinson (Mountain View, California) flow cytometer and CellQuest software. Sub-G1 phase represents the apoptotic population.

Western blot and antibodies. Protein lysate was prepared with 50 mmol/L Tris buffer (pH 7.4) containing 10 mmol/L EDTA, 1% Triton X-100, and 1 mmol/L sodium orthovanadate, centrifuged at 30,000 × g for 30 minutes, and stored at −80°C until analysis. A 30% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed, and protein samples were transferred to a nitrocellulose membrane. Membranes were nonspecifically blocked in 5% nonfat dry milk in Tris-buffered saline (TBS) for 1 hour and incubated with the primary antibody at 4°C overnight. After washing, the membranes were incubated with the secondary antibody for 1 hour. The following antibodies were used: anti-β-catenin, anti-p53, anti-Bcl-xL, anti-Akt, anti-phospho-Akt, and anti-cyclin D1 (Cell Signaling Technology, Beverly, Massachusetts).
X-100, and protease inhibitors. Proteins were separated by SDS-PAGE gel. Anti-β-catenin monoclonal antibody was purchased from BD Biosciences PharMingen (Mississauga, Ontario). The antibodies for caspase-3, caspase-6, Bcl-2, Bax, Bcl-xL, Akt, and phosphorylated Akt were purchased from Cell Signaling Technology (Beverly, MA). Cytochrome c isolation kit from Cell Signaling Technology was used to measure cytosolic cytochrome c. Anti-mouse and anti-rabbit immunoglobulin G peroxidase (Jackson, Bar Harbor, ME) were used as secondary antibodies.

**Results and Discussion.**

We stably transfected RKO colonic epithelial cells with cDNA encoding a mutant form of β-catenin, S37A, to create an RKO β-catenin–overexpressing cell line (RKOβ-cat). This mutant is resistant to phosphorylation-dependent ubiquitination and degradation, resulting in its stabilized expression (16). Western blot showed that the protein level of β-catenin was much higher in RKOβ-cat cells than in the parental cell line, RKO (Fig. 1A). To test the relationship between β-catenin overexpression and nitric oxide–induced apoptosis, RKO and RKOβ-cat cells were exposed to the nitric oxide donors diethylenetriamine-NONOate (DETA-NONOate; ref. 17) and sodium nitroprusside. Exposure to DETA-NONOate caused concentration- and time-dependent apoptosis as measured by flow cytometry after staining with propidium iodide (Fig. 1B). Overexpressed β-catenin in RKOβ-cat cells significantly blocked nitric oxide–induced cell death (Fig. 1B). Sodium nitroprusside (0.5 mmol/L) also induced apoptosis in RKO cells (10.23 ± 2.29%), which could be significantly blocked by β-catenin in RKOβ-cat cells (1.64 ± 0.35%; P < 0.05).

Exposure to DETA-NONOate resulted in the activation of caspase-3 and caspase-6 and triggered an increase in cytoplasmic cytochrome c in RKO cells, but not in RKOβ-cat cells (Fig. 1C and D). These data show that, in the RKO cell line, nitric oxide caused apoptosis mediated by the cytochrome c-caspase pathway and that β-catenin overexpression confers protection against nitric oxide–induced apoptosis.

Different lines of evidence show that p53 is involved in nitric oxide–induced apoptotic cell death (18). In our studies, DETA-NONOate induced an accumulation of p53 in RKO cells as early as 12 hours after treatment, which could not be blocked by β-catenin (Fig. 2A). In RKO-E6 cells, an RKO derivative characterized by a loss of function of p53 (Fig. 2B; ref. 19), nitric oxide–induced apoptosis was significantly blocked (Fig. 2C). Our data not only show that activation of p53 preceded the induction of apoptosis by the nitric oxide donor but also suggest that p53 is required for the early, but not later, stages of apoptosis. This contention is supported by the observation that p53 activation had abated (Fig. 2A), whereas apoptosis was still elevated at 72 hours after exposure to DETA-NONOate (Fig. 1B). It has previously been shown that p53 might be an early player in colitis-related colon cancer (20). Our studies add further support to the hypothesis that p53 plays an important role in the clonal selection process during chronic inflammation–induced cancer.

Previous studies have shown interactions between p53 and β-catenin. Excessive β-catenin partially inhibits mdm2-mediated proteolysis of p53 (21), whereas activated p53 down-regulates β-catenin through the ubiquitin-proteasome system, a process that requires active glycogen synthase kinase-3b (22). In the present study, we showed both an elevated basal level of p53 in RKOβ-cat cells (Fig. 2B) and a partial decrease of β-catenin after treatment with nitric oxide donor in RKO cells, but not in RKOβ-cat cells (Fig. 2D). In RKO-E6 cells, loss of function of p53 partially blocked the degradation of β-catenin induced by p53 (Fig. 2D). These data suggest that nitric oxide–induced degradation of β-catenin is mediated by both p53-dependent and p53-independent pathways, and that β-catenin blocks apoptotic cell death after the activation of p53.

To further elucidate the mechanism underlying the above observations, we assessed the changes in expression of Bcl-2 family proteins and Akt. Bcl-2 family proteins are known to regulate mitochondrial release of cytochrome c during the

**Figure 1.** Overexpression of β-catenin blocked nitric oxide–induced apoptosis in RKO cells. A, Western blot showing the protein level of β-catenin in RKO and RKOβ-cat cells, which were transfected with β-catenin S37A. B, RKO and RKOβ-cat cells were treated with nitric oxide donor, DETA-NONOate, and then were analyzed by flow cytometry for apoptosis. C, cells were treated with DETA-NONOate at 0, 0.25, or 0.5 mmol/L for 48 hours, and then were collected to measure the level of cleaved caspase-3 and caspase-6 and the cytosolic cytochrome c (D) by Western blot. Columns, mean; bars, SE. *P < 0.05, versus corresponding control groups without treatment. †, P < 0.05, versus corresponding RKO groups with the same treatment.
initiation of apoptosis. Bcl-xL, as a potent repressor of apoptosis, can protect activated macrophages from nitric oxide–induced apoptosis (23). Exposure of RKO cells to the nitric oxide donor did not result in any change in the protein levels of Bcl-2, Bax, and Bak as determined by Western blot (Fig. 3A). However, the nitric oxide donor significantly decreased Bcl-xL levels, which was blocked by overexpression of β-catenin through both Bcl-xL– and Akt-related pathways.

Taken together, our data show that nitric oxide induces accumulation of p53, decrease in β-catenin and Bcl-xL, release of cytochrome c from mitochondria, and then apoptosis. Either inactivated p53 or constitutively activated β-catenin could block nitric oxide–induced apoptosis. During chronic inflammation, where inducible nitric oxide synthase is activated and a high

Figure 2. Role of p53 in nitric oxide–induced cell death. A, RKO and RKOβ-caten cells were collected 12, 24, 48, and 72 hours after treatment with or without DETA-NONOate (0.5 mmol/L). The level of p53 was detected by Western blot. Cells were treated with DETA-NONOate at 0, 0.25, or 0.5 mmol/L for 48 hours, and then were collected to analyze p53 by Western blot (B), apoptosis by flow cytometry (C), and β-catenin by Western blot (D). Columns, mean; bars, SE. *, P < 0.05, versus corresponding control groups without treatment. #, P < 0.05, versus corresponding RKO groups with the same treatment.

Figure 3. β-Catenin blocked nitric oxide–induced apoptosis through Bcl-xL and Akt. The cells were treated with DETA-NONOate for 48 hours and then samples were collected for Western blot. A, Bcl-2 family proteins; B, Bcl-xL; C, phosphorylated Akt. Columns, mean; bars, SE. *, P < 0.05, versus RKO cells with the same treatment.
tissue level of nitric oxide is maintained (4), cells expressing wild-type p53 and low level β-catenin are subject to nitric oxide–induced apoptosis. However, when either p53 or β-catenin regulatory pathway mutates, resulting in excessive cytosolic β-catenin, cells become resistant to nitric oxide–induced apoptosis and survive with damaged DNA. The consequence is the clonal expansion of these cells which, with time and further mutation, may undergo transformation to cancer.

References


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