**G1 Delay in Cells Overexpressing Prostaglandin Endoperoxide Synthase-2**

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**Abstract**

Colorectal cancer is the second leading cause of death from cancer in the United States. Continuous use of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) has been shown to reduce the risk of colorectal cancer in humans by 40–50%. Patients with familial adenomatous polyposis who take NSAIDs, such as sulindac, undergo a regression of intestinal adenomas. Rodents exposed to carcinogens that cause colon cancer have a 50–60% reduction in the size and number of colonic tumors when treated continuously with NSAIDs. One common target for these drugs is prostaglandin endoperoxide synthase, also referred to as cyclooxygenase (COX). We and others have shown recently that COX-2 is inducible by a variety of factors, which include cytokines, growth factors, and tumor promoters (1). COX-2 was identified initially by many groups as a member of a class of genes referred to as “immediate early” or “early growth response” genes (2–6). These genes are induced rapidly and transiently following growth factor or phorbol ester stimulation of quiescent cells (7–9). The functions of these genes are quite diverse and range from nuclear transcription factors (c-fos and jun-B) to cytokines. The precise role many of these genes play in regulation of cellular responses to growth stimuli and tumor promoters has not been defined clearly. Continuous NSAID use in humans has been shown to reduce the risk of colorectal cancer by ~50% (10–14), but the molecular basis for the chemopreventive effects of these drugs remains unknown. Studies carried out in rodents have shown a marked reduction in carcinogen-induced colon cancer in animals treated with NSAIDs (15–18). We and others have shown an increased level of COX-2 expression in human colorectal adenocarcinomas (19–21). Our laboratory also has demonstrated recently that adhesion to selected extracellular matrix proteins is enhanced and apoptosis is inhibited in intestinal epithelial cells that are programmed to overexpress the COX-2 gene (22). These phenotypic changes, which are related to overexpression of COX-2 and reversible by treatment with NSAIDs, may alter the tumorigenic potential of intestinal epithelial cells.

**Regulation of epithelial cell cycle progression** as it relates to malignant transformation has become the focus of intense interest over the past decade (23–25). Late in G1, the cell commits to undergo chromosomal DNA replication. There is a restriction point late in G1, at which time the cells no longer require growth factors to enter the S-phase. An accumulation of unstable proteins is required to pass the restriction point (26). It has been hypothesized that mammalian G1 cyclins, which regulate one or more cdks, drive cell cycle progression in a rate-limiting manner. It has been shown clearly that overexpression of D-type cyclins can shorten the G1 interval (27–30), and these studies provide direct evidence of the importance of D cyclins in the regulation of G1 progression. We report here that in intestinal epithelial cells programmed to overexpress COX-2, there is a marked delay in progression through G1. Additionally, we observe a marked decrease in cyclin D1 expression and Rb kinase activity associated with the G1 delay in COX-2-overexpressing cells.

**Materials and Methods**

**Cell Culture.** Rat intestinal epithelial (RIE-1) cells were a gift from K. D. Brown (Cambridge Research Station, Babraham, Cambridge, United Kingdom). Cells were grown on plastic dishes in DMEM supplemented with 10% fetal bovine serum (lot 11151032; Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, 100,000 units/liter penicillin G, and 100,000 μg/liter streptomycin sulfate.

**Stable Transfection.** A 2.1-kb Smal-EcoRV fragment containing the open reading frame for a polypeptide of 604 amino acids of rat COX-2 was isolated and blunt-end cloned into the Smal site of the eukaryotic expression vector pcB7 (a gift from Bob Coffey, Vanderbilt University). In this vector, transcription of the cDNA is controlled by the cytomegalovirus promoter. This vector also contains a bacterial hygromycin resistance gene expression cassette, which allows for selection in hygromycin B. S- and AS-oriented expression vectors were prepared. These expression vectors were transfected into a nontransformed rat intestinal epithelial cell line (RIE-1) using a Lipofectin (GIBCO-BRL, Gaithersburg, MD) transfection method (2), and the cells were selected in medium containing hygromycin B (225 units/ml). Total RNA was prepared from the cells transfected with sense-oriented expression and evaluated using Northern blot slot blot analysis. Five clones (S10, S99, S123, S132, and S146) expressing the highest level of COX-2 RNA were identified from 225 initial clones and expanded. We characterized all five clones and found that they all exhibited the identical phenotypic and biochemical alterations. Therefore, the results of our studies using the RIE-S10 clone are presented here. The RIE cells transfected with the antisense expression vector (RIE-AS) were evaluated in a similar manner.

**Northern Blotting.** RNA and Northern blotting was carried out essentially as described (19, 31).

**Immunoblotting.** The cells were lysed by for 30 min at 4°C in radioluminoprecipitation assay buffer [150 mM NaCl, 1% Nonidet P-40, and 50 mM...
containing 10% fetal bovine serum, and the studies were carried out in

was added to each well for 2 h. Trichloroacetic acid-precipitable material was

triplicate. After various times following serum addition, 1μCi [3H]thymidine

CA) was used at a 1:2000 dilution; and p21 (Oncogene Science, Uniondale,

Galveston, TX) and human cdk4 (Santa Cruz Biotechnology, Inc., Santa Cruz,

globulin as a secondary antibody (1:5000 dilution) for 1 h. After three addi-

rabbit antisera to human cyclin D1 (Upstate Biotechnology, Inc., Lake Placid,

NY) was used at a 1:500 dilution; polyclonal rabbit antisera to mouse cyclin

D3 (a gift from Aubrey Thompson, University of Texas Medical Branch,

Rb KO (a gift from Ed Leof (Mayo Clinic, Rochester, MN). Primary antibodies

system (Amersham, Arlington Heights, IL) and exposed to XAR5 film (East

Tris (pH 8.0)) containing 10μg/ml aprotinin, 10μg/ml leupeptin, 1μg/ml

phenylmethylsulfonylfluoride. Centrifuged cell lysates (50μg) were denatured

and fractionated on 11.25% polyacrylamide gels

Tris (pH 6.8), 0.5% β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue,

10% glycerol] and boiled for 5 min, then analyzed by SDS-PAGE and

autoradiography. The glutathione S-transferase-Rb fusion protein was obtained

from Santa Cruz Biotechnology, and the Rb fusion fragment consists of amino

acids 769–921 of the carboxyl-terminal domain of the mouse pRb. The cdk4

antiseras was a kind gift from Ed Leof (Mayo Clinic, Rochester, MN).

Results

To investigate the role of COX-2 expression in intestinal epithelial
cell cycle regulation, RIE cells were transfected permanently with the
eukaryotic expression vector pCB7 containing the COX-2-coding

region (2.2 kb) in either the S or AS orientation. Northern and

DNA Synthesis. DNA synthesis was measured by [3H]thymidine incorpo-

ration into trichloroacetic acid-precipitable material (31). RIE cells were plated

in multiwell dishes, grown to 80% confluence, and then placed in serum- and

growth factor-free medium for 72 h. Then, the cells were exposed to DMEM

containing 10% fetal bovine serum, and the studies were carried out in

normalized to 1 X 10^6 cells.

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Western blotting analyses were carried out to determine relative levels of COX-2 mRNA and protein in these cells. COX-2 expression levels in RIE-S cells were compared with RIE-P cells in the presence or absence of growth stimulation by the addition of 10% serum to the cell culture medium. As shown in Fig. 1A, there are two mRNA bands present in the serum-treated RIE-S cells, which hybridize to the COX-2 cDNA probe: a 4.5-kb mRNA, which corresponds to the endogenous transcript; and a 2.2-kb band, which corresponds in size to the transfected COX-2 gene. In the RIE-AS cells, only the 2.2-kb antisense mRNA is present, indicating that the presence of the antisense transcript inhibits the appearance of the endogenous COX-2 mRNA. The response of the RIE-P cells to serum stimulation is shown in Fig. 1A (far right panel), and only the 4.5-kb transcript is present in these nontransfected cells. COX-2 protein levels were determined by Western blotting and are shown in Fig. 1B. These results indicate that the level of COX-2 protein correlates well with the level of mRNA and that COX-2 protein levels in the RIE-S cells are equivalent to levels seen transiently following serum induction. Also, these results demonstrate that the antisense construct inhibits the expression of the COX-2 protein and mRNA in RIE-AS cells effectively.

We observed empirically that the RIE-S cells grow more slowly than the RIE-AS and RIE-P cells. Then, we examined progression through G1 in the RIE-P, RIE-AS, and RIE-S cells plated on plastic dishes. Our results, shown in Fig. 2A, demonstrate that the length of time required to enter the S-phase, as measured by [3H]thymidine incorporation into DNA, was reduced from 12 h in RIE-P cells to 8 h in the RIE-AS cells and occurred between 20 and 24 h in the RIE-S cells. These results were confirmed by cell sorting analysis (Fig. 2B). This series of experiments demonstrates clearly a 2–3-fold increase in the time required for progression through G1 in the cells that overexpress the COX-2 gene constitutively.

Work by several investigators has demonstrated that the D-type cyclins are “delayed-early” genes that regulate the rate of progression through G1 (27–30). Cyclin D1 is a critical target of proliferative signals following growth factor stimulation of quiescent cells, and inhibition of its expression can cause a G1 arrest. Inhibition of intestinal epithelial cell growth by TGF-β is associated with down-regulation of cyclin D1, with little effect on cdk4 or cyclin D3 levels (32). Therefore, cyclin D1 seems to perform a crucial role in regulating the progression of intestinal epithelial cells through the first gap of the cell cycle. Next, we examined the levels of cell cycle-regulated proteins in exponentially growing RIE-P, RIE-S, and RIE-AS cells. Our results, shown in Fig. 3, demonstrate that cyclin D1 protein levels are barely detectable in asynchronous RIE-S cells in the midlog growth phase but easily detected in the RIE-P and RIE-AS cells, whereas cyclin D3 levels were equivalent in all three cell lines. The level of cdk4 was reduced by 50% in the RIE-S cells, whereas p21 levels seemed to be slightly elevated compared with levels seen in RIE-S cells. From these results, we conclude that cyclin D1 expression was decreased in intestinal epithelial cells programmed to overexpress COX-2.

Then, we explored the temporal pattern for changes in cyclin D1, cdk4, and p21 levels following serum stimulation of quiescent RIE-P, RIE-S, and RIE-AS cells. These results, shown in Fig. 4, indicate that cyclin D1 is expressed abundantly in serum-deprived RIE-AS cells, and its levels do not change significantly following serum stimulation. The level of cyclin D1 is very low in serum-deprived RIE-S cells, its induction is markedly delayed, and the level is reduced compared with the pattern seen for the RIE-P cells. Induction of cdk4 and p21 is also delayed in the RIE-S cells compared with the other two cell lines, and
suggest that overexpression of COX-2 may confer a survival advantage to cells by slowing cell cycle progression, preventing apoptosis, and enhancing adhesion to the extracellular matrix. Prolonged survival of abnormal cells can favor tumor progression and facilitate the accumulation of sequential genetic mutations which would result in tumor promotion. Our results are consistent with this hypothesis and suggest a link between COX-2 overexpression, prolongation of G1, and inhibition of apoptosis in intestinal epithelial cells. Studies are underway to determine the effect of selective COX-2 inhibitors on cyclin protein levels in COX-2-overexpressing intestinal epithelial cells.

**References**


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