Altered expression of cyclin D1 and cyclin-dependent kinase 4 in azoxymethane-induced mouse colon tumorigenesis

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Alterations in the expression of the cell cycle regulators, cyclin D1 and cyclin-dependent kinase 4 (Cdk4), have been implicated in malignancies of both humans and experimental animal models. We hypothesize that altered expression of cyclin D1 and Cdk4 may also be involved in mouse colon tumorigenesis induced by the chemical carcinogen, azoxymethane (AOM). In the present study, SWR/J mice were given AOM by i.p. injection at a dose of 10 mg/kg once a week for 8 weeks, and colonic tissue and tumors were isolated 18 weeks later. The expression and localization of cyclin D1 and Cdk4 were examined by reverse transcription–polymerase chain reaction (RT–PCR) and immunohistochemical analyses. Cyclin D1 and Cdk4 mRNA levels in tumor samples were increased 1.3-fold ($P < 0.01$) and 1.2-fold ($P < 0.01$), respectively, when compared with control mouse colon tissue. Control colon epithelium was uniformly negative for cyclin D1 immunoreactivity, whereas minimal Cdk4 nuclear staining was confined to the lower portion of the crypts within the control tissue. Both cyclin D1 and Cdk4 immunoreactive cells were markedly increased in preneoplastic lesions and in adenomas isolated from AOM-treated mice. Furthermore, some morphologically normal colon crypts from AOM-treated mice showed positive cyclin D1 immunoreactivity. These findings suggest that overexpression of cyclin D1 and Cdk4 occurs early in the AOM-induced mouse colon tumorigenesis and may contribute to tumor progression in this model.

Introduction

Colorectal cancer is the third most common malignancy worldwide and the second leading cause of cancer deaths in the USA (1,2). Administration of the methylating carcinogen, 1,2-dimethylhydrazine (DMH), and its active metabolite, azoxymethane (AOM), produces colon tumors in susceptible rodent strains that exhibit pathological features similar to those seen in the human disease (3–5). In addition, a number of genetic alterations associated with human colorectal cancer have been found in preneoplastic lesions and in tumors of the colon of these animals after exposure to DMH or AOM (6–10). Thus, the rodent model of colon carcinogenesis provides a useful experimental system for studying molecular and morphological changes associated with human colorectal cancer.

Cyclins are positive regulators of cell cycle progression. They function by forming a complex with and activating a class of protein kinases, i.e. cyclin-dependent kinases (Cdks), which are essential for cell cycle transitions (11–14). Cyclin D1 binds to and activates its major catalytic subunits, Cdk4 and Cdk6, which regulate $G_1$ phase progression and $G_1/S$ transition through phosphorylation of the Rb tumor suppressor protein as well as two Rb-related proteins p107 and p130 (11–14). As the major regulatory events leading to cell proliferation occur in the $G_1$ phase of the cell cycle, altered expression of $G_1$ cyclins and their Cdks may be an important step in oncogenesis (11–14). Studies in human cancer have shown that $G_1$ cyclins and their Cdks are the most consistently altered cell-cycle regulatory proteins and, among them, an accumulating body of evidence suggests that deregulated expression of cyclin D1 and Cdk4 is associated with malignancy.

Cyclin D1 overexpression has been reported in a variety of human cancers including colon cancer (15–22), and antisense to cyclin D1 inhibits the growth and tumorigenicity of human colon cancer cells (23). Overexpression of Cdk4 has been observed in a subset of malignant human glial tumors and in human sarcomas (24,25). Recently, concurrent overexpression of cyclin D1 and Cdk4 has been detected in adenomatous polyps of human colon, as well as in intestinal adenomas from multiple intestinal neoplasia (Min) mice (26). However, how early these alterations occur in colon tumorigenesis remains to be determined both in humans and in rodent models.

Although a number of genetic changes have been detected in the DMH- and AOM-induced colon tumor model (7–10), it is unclear whether alterations in the cell cycle regulatory proteins are also involved in the tumorigenic process. A further examination of the molecular events associated with the cell cycle regulation would help to elucidate the molecular mechanisms underlying DMH- and AOM-induced colon tumorigenesis. In addition, it may also be possible to identify potential biomarkers associated with the stepwise tumor progression in this model. The objective of the present study was to test our hypothesis that alterations in the cell cycle regulatory proteins, cyclin D1 and Cdk4, may be involved in the AOM-induced mouse colon tumorigenesis. The identification of these alterations at early stage may enable their use as molecular biomarkers to predict tumor development, and as surrogate endpoint biomarkers for cancer chemoprevention studies in this model.

Materials and methods

Animals and treatment

Five-week-old male SWR/J mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and housed in a controlled environment with a 12 h
light−dark cycle. Mice were provided with Purina laboratory rodent chow and water ad libitum. After an acclimation period of 1 week, mice were treated with AOM dissolved in saline by i.p. injection at a dose of 10 mg/kg body wt once per week for 8 weeks. Control animals received saline as vehicle controls. AOM-treated and vehicle control mice gained body weight at similar levels throughout the course of the study.

Tissue sample preparation

Eighteen weeks after the last injection of AOM, mice were killed and their colon was flushed with ice-cold phosphate-buffered saline (PBS). Colon tumors and grossly normal-appearing colonic tissue from the AOM-treated group were isolated. Normal colon tissue from the control group was also obtained. The tissues were divided into two macroscopically similar portions for RNA isolation and paraffin embedding. The portion for RNA isolation was immediately frozen in liquid nitrogen and stored at −80°C. The other portion was fixed in 10% neutral buffered formalin for 12 h, followed by paraffin embedding for subsequent histopathological examination.

RNA isolation and reverse transcription−polymerase chain reaction (RT−PCR)

Using TRizol reagent (Gibco BRL, Gaithersburg, MD), total cellular RNA was isolated from previously frozen tissues according to the manufacturer’s instructions. All RNA samples were analyzed for integrity of 18S and 28S rRNA by ethidium bromide staining of 1 µg of total RNA resolved by electrophoresis on 1.2% agarose formaldehyde gels. RT−PCR analysis was performed as described previously (27,28). Briefly, RNA was incubated at 60°C for 10 min and chilled to 4°C before being reverse transcribed. Reverse transcription of 2 µg of total RNA using random hexamers was performed in a volume of 20 µl for 60 min at 42°C containing 5 U of AMV reverse transcriptase (Gibco BRL), 10 mM Tris HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl2, 0.2 mM dATP, dGTP, dCTP and 1.25 U of Taq DNA polymerase (Gibco BRL) in a final volume of 50 µl. Water and reverse transcriptase minus reactions were run as negative controls.

The primers used for amplification of cyclin D1 were, sense 5′-GTCAGACCAATCTTCTTCAACGC-3′ and antisense 5′-GGCGGCGAGTCTCCTTGAAC-3′; primers used for amplification of Cdk4 were, sense 5′-CTTCCGTAGACACAGGCT-3′ and antisense 5′-GGTACGATTTCCAGTAGC-3′; and primers used to amplify HPRT were, sense 5′-GAAACGCAGTGTCCGCT-3′ and antisense 5′-CCAGCAAGCTTGCACCAAC-3′, according to the published sequences or primer sets (29−32).

Amplification of cyclin D1 was performed as described (29) in 24 cycles. For amplification of Cdk4 and HPRT, the reaction mixture was first heated at 95°C for 5 s, 45 s for 45 s and 72°C for 1 min, followed by an incubation for 10 min at 72°C. The number of amplification cycles was previously determined to avoid the ‘plateau effect’ associated with increased numbers of PCR cycles (33). PCR products of each specific gene were electrophoresed through a 1.8% agarose gel in 0.5× TBE buffer. For quantitation of PCR products, the intensity of ethidium bromide luminescence was measured using a Bio-Rad Image Analysis System, Gel Doc 1000, with Molecular Analyst software (Bio-Rad, Hercules, CA). The intensity of each cyclin D1 and Cdk4 band was normalized to the intensity of the corresponding HPRT control bands. Statistical analysis was performed using Student’s t-test.

Immunohistochemical staining

Paraffin-embedded tissue sections (4 µm) were deparaffinized and rehydrated. Endogenous peroxidase activity was ablated with 1% hydrogen peroxide in PBS. Tissue sections were then heated in 10 mM sodium citrate, pH 6.0, in a microwave oven for 10 min to expose the antigens. Goat serum (10%) was used to suppress non-specific protein binding. Tissue sections were incubated at 4°C overnight with rabbit polyclonal anti-cyclin D1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:3000 dilution or rabbit polyclonal anti-Cdk4 antibody (Santa Cruz Biotechnology) at 1:10 000 dilution. The sections were then washed and incubated with biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA) at room temperature for 30 min. After washing, the sections were incubated with avidin–biotin peroxidase complex at room temperature for 30 min using the VECTASTAIN Elite ABC kit (Vector). After color development with 3,3′-diaminobenzidine as the substrate, sections were counterstained with Harris’ acid hematoxylin. To demonstrate the specificity of the immunostaining, the primary antibodies were replaced with similar protein concentrations of normal rabbit IgG.

Results

Histopathological examination

Hematoxylin and eosin stained sections were examined microscopically to confirm the histology of each type of tissue. The tumor samples used in this study were benign exophytic adenomas. Colonic mucosa obtained from AOM-treated mice exhibited various histopathological changes, ranging from hyperplasia to dysplasia. Colonic mucosa from the control mice showed normal histology.

Semi-quantitative RT−PCR analysis of cyclin D1 and Cdk4 mRNA expression

Twenty adenomas from 10 AOM-treated mice and 12 specimens of colonic mucosa from 12 control mice were used in this study. Because of sample size requirement, we were not able to perform RT−PCR analysis in any type of preneoplastic lesions. RT−PCR analysis of RNA samples isolated from the tissues using primers specific for cyclin D1, Cdk4 and the house-keeping gene, HPRT; produced DNA fragments of the expected sizes (Figure 1). The cyclin D1 and Cdk4 mRNA levels in each tissue type, quantified by scanning the bands using an image analysis system and normalized to HPRT levels, are shown in Figure 2. Both cyclin D1 and Cdk4 mRNA levels were increased in tumor samples compared with the mRNA levels in normal mucosa. Overall, cyclin D1 mRNA levels were elevated 1.3-fold (P < 0.01) and Cdk4 mRNA levels were increased 1.2-fold (P < 0.01) in adenomas when compared with the mRNA levels in normal mucosa. The samples that showed increased levels of cyclin D1 mRNA also demonstrated elevated levels of Cdk4 mRNA (Figure 1).

Immunohistochemical studies of cyclin D1, Cdk4 protein expression and localization

To examine whether increased cyclin D1 and Cdk4 mRNA expression was accompanied by increased expression of their protein products, and to analyze their cellular and subcellular localization within the tissue architecture, immunohistochemical analyses were performed. Figures 3 and 4 show representative stained sections from vehicle-control colonic epithelium, AOM-induced preneoplastic lesions and adenomas. Control colonic epithelium was uniformly absent of cyclin D1 positive cells. In contrast, cyclin D1 immunostaining was evident in preneoplastic lesions and in adenomas from AOM-treated mice. Positively stained cells for cyclin D1 were most prevalent in adenomas. Furthermore, some morphologically
normal colon crypts from AOM-treated mice showed positive cyclin D1 immunoreactivity. In control colon epithelium, positive Cdk4 immunostaining was observed but was mostly restricted to the lower regions (proliferative zones) of the crypts. The number of Cdk4-positive cells increased in preneoplastic lesions and adenomas, with the Cdk4 immunoreactivity most abundant in adenomas. In preneoplastic lesions and adenomas, cyclin D1 and Cdk4 positive cells were observed throughout the entire length of the colonic epithelium including the upper one-third of the crypts. Both cyclin D1 and Cdk4 immunoreactivity was predominantly confined to the nuclei, and the staining pattern and intensity of these two proteins correlated well with each other, as assessed by staining consecutive tissue sections. Control sections incubated with normal rabbit IgG showed no staining (data not shown).

Discussion

In the present study, we examined cyclin D1 and Cdk4 expression in an AOM-induced SWR/J mouse colon model. AOM, an active metabolite of DMH, offers advantages over DMH, including enhanced stability in dosing solution and its greater effectiveness in producing colon tumors in rodents at lower doses (34,35). In addition, it is not metabolized to expired intermediates (e.g. azomethane) that pose a hazard to researchers. Thus, AOM has been used extensively to study colon tumorigenesis in both mouse and rat models (4,5,7,10).

In the study presented here, cyclin D1 and Cdk4 mRNA levels were measured by RT–PCR analysis in normal mouse colon tissues and adenomas. In addition, the expression and localization of protein products were analyzed by immunohistochemistry. Sample size requirements for obtaining sufficient amounts of RNA for RT–PCR analysis did not allow detection of either cyclin D1 or Cdk4 mRNA levels in the preneoplastic lesions. However, using immunohistochemical techniques, we were able to detect cyclin D1 and Cdk4 protein expression in those lesions from paraffin-embedded tissue sections.

We observed increases of cyclin D1 and Cdk4 mRNA levels in adenomas when compared with normal colonic mucosa. Immunohistochemical studies confirmed these observations and correlated well with the findings at the mRNA level. There was a sequential increase of cyclin D1 and Cdk4 protein expression from normal colonic epithelium, to preneoplastic lesions, and ultimately to adenomas. In addition to the marked increase of cyclin D1 and Cdk4 expression in the preneoplastic lesions and adenomas, there was an upward shift of cyclin D1 and Cdk4-positive cells to the upper one-third of the crypt compartment including the luminal surface, in these lesions. It is noteworthy that some morphologically normal crypts in AOM-treated mice showed overexpression of cyclin D1 protein. Alterations in the production of other proteins such as M1 antigens, mucins and certain enzymes have also been reported in histologically normal colon mucosa of rodents after DMH or AOM treatment (4,36,37). The present study indicates that increased expression of cyclin D1 and Cdk4 occurs relatively early in AOM-induced mouse colon tumorigenesis and is associated with tumor development. In the DMH- or AOM-induced colon tumor model, adenomas may progress to carcinomas, and carcinomas may also derive de novo from dysplastic epithelium (4,38). Further studies using alternative animal treatment protocols to generate a large quantity of carcinomas are needed to determine if cyclin D1 and/or Cdk4 expression is further increased in carcinomas induced by AOM.

Overexpression of cyclin D1 and Cdk4 has been associated with increased cell proliferation, as evidenced by increased cell proliferation indices (26,39). Since cyclin D1 and Cdk4 are essential for controlling G1 phase progression and the onset of DNA replication, overexpression of these two genes may cause abnormalities in cell growth control and proliferation rate. Our observations that cyclin D1 and Cdk4 were overexpressed in hyperproliferative epithelium such as preneoplastic lesions and adenomas supports this notion. Indeed, transgenic mice with cyclin D1 overexpression showed increased cell proliferation in targeted tissues (39). Thus, the aberrant localization of cyclin D1 and Cdk4 positive cells beyond the normal proliferative compartment of the colonic crypts observed in this study may reflect the expansion of the proliferative zone in these lesions. However, it is intriguing that cyclin D1 immunoreactivity was undetectable in the normal colon crypts from control animals, although cell proliferation is active at the base of the crypts. Similar findings have been observed in the human colon and in the Min mice intestine (16,26). As suggested previously, it is possible that cyclins D2 or D3 are more important in activating Cdk4 in the normal intestinal crypts (16). It is also possible that the expression level of cyclin D1 protein is low in the normal crypts, which is undetectable by the method employed in these studies.

Cyclin D1 and Cdk4 overexpression has been found in adenomatous polyps and adenocarcinomas in human colon (16,21,26). The observation that overexpression of these genes occurred in adenomatous polyps indicates an early role of cyclin D1 and Cdk4 in human colon carcinogenesis. However, the mechanism(s) underlying cyclin D1 and Cdk4 overexpression in colon cancer remains to be elucidated. Overexpression of cyclin D1 has been associated with DNA amplification in some, but not all human cancers (18,40,41),
and amplification of cyclin D1 does not occur in human colon cancer (16,21,42). Recently, it has been shown that oncogenic ras protein can induce the expression of cyclin D1 (43–45), and cyclin D1 is a critical target of the ras signaling cascade regulating cell cycle progression (46). Activation of the K-ras oncogene is one of the most common genetic alterations in human colon cancer, and occurs at an early stage during colon carcinogenesis (47–49). K-ras activation has also been detected in rat and mouse colon aberrant crypt foci, adenomas and carcinomas after DMH or AOM exposure (7,50–52). Furthermore, K-ras activation has been detected in grossly normal mucosa after DMH or AOM treatment (52,53). These studies suggest that, as in human colon cancer, K-ras activation may be one of the earliest events in this murine colon tumor model, and overexpression of cyclin D1 may be accounted for by activated K-ras oncogene. The activation status and temporal expression of the K-ras oncogene in the mouse colon with the same animal treatment protocol used in this study is currently under investigation. Amplification of Cdk4 has also been detected in some human cancers with concurrent overexpression of the Cdk4 gene (24,25). However, amplification of Cdk4 in human colon cancer has not been reported. It has been shown that increased cyclin D1 gene expression can induce Cdk4 activity (54), a finding that is consistent with the
role of Cdk4 as a major catalytic partner of cyclin D1. A direct evidence that cyclin D1 can induce Cdk4 gene expression comes from a recent transgenic mouse study in which cyclin D1 overexpression induced Cdk4 expression in targeted tissues (39). It is conceivable, therefore, that increased expression of Cdk4 observed in the present study may be a consequence of cyclin D1 overexpression. On the other hand, recent studies have suggested that cyclin D1 and Cdk4 are important targets of transforming growth factor-β (TGF-β) regulatory pathways (55–57). Recently, it has been shown that decreased expression of TGF-β type II receptor is associated with overexpression of cyclin D1 and Cdk4 in a Min mouse model (29). Furthermore, it has been shown that TGF-β 1 expression is reduced in ACFs induced by DMH in rat colon (58). It is possible that altered TGF-β pathways liberate cells from TGF-β imposed negative regulation, thus promoting cyclin D1 and Cdk4 expression. In fact, alterations in TGF-β and its receptors are common findings in human colon carcinogenesis (59–61). The correlation between TGF-β regulatory pathways and the expression of cyclin D1 and Cdk4 remains to be determined in the AOM-induced mouse colon model.

In conclusion, our observations in the present study are consistent with other evidence that indicates that overexpression of cyclin D1 and Cdk4 are frequent alterations that occur during colon tumorigenesis in both humans and rodents. In addition, our findings suggest that altered expression of cyclin D1 and Cdk4 occurs early in the AOM-induced mouse colon tumorigenesis, and may participate in tumor progression. Further studies are needed to delineate the mechanism(s) of these alterations.

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References


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