Activation of Adenomatous Polyposis Coli (APC) Gene Expression by the DNA-alkylating Agent N-Methyl-N′-nitro-N-nitrosoguanidine Requires p53*

(Received for publication, July 17, 1997, and in revised form, September 19, 1997)

Satya Narayan‡§ and Aruna S. Jaiswal‡

From the ‡Sealy Center for Oncology and Hematology and §Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77555-1048

Development of colon cancer is a multistep process frequently involving mutations in both the APC and p53 tumor suppressor genes. In this study we treated the HCT-116 colon cancer cell line with alkylating agents including N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), which is known to cause colon cancer in animals, and examined the expression of both APC and p53 genes. Exposure of cells with MNNG caused an 8–12-fold increase in the level of APC mRNA and protein. APC induction was shown to result from increased nuclear transcription of the APC gene and correlated with a concomitant increase in the p53 protein level after MNNG treatment. A necessary role for p53 in APC gene regulation is supported by the failure of MNNG to induce APC expression in cell lines either expressing very low levels of p53 (HeLa cells) or no p53 (K562 erythroleukemia cells). The overexpression of wild-type p53 gene into HCT-116 cells mimicked the effect of MNNG-induced expression of APC mRNA. A direct causal role for p53 in APC gene regulation was further evaluated by transfecting the wild-type p53 gene into K562 cells and observing a 5-fold increase in the APC gene expression. These results support a model featuring a direct link between p53 and APC in response to alkylation-induced DNA damage and suggest a novel role for p53 in a stress-response pathway involving APC.

Intestinal cells are constantly exposed to DNA-damaging agents from dietary toxins (1, 2). The resulting DNA damage, if not efficiently repaired, may result in genomic instability, leading to malignant transformation. The development and progression of colon cancer is a multistep process in which growth control mechanisms are impaired progressively. Mutations of the adenomatous polyposis coli (APC) tumor suppressor gene, the Ki-ras oncogene, the deleted in colorectal cancer (DCC) gene, the p53 gene, and DNA mismatch repair (MMR) genes play important roles at different stages of colorectal carcinogenesis (for review, see Ref. 3). Among these, mutation of the APC gene is an early event in familial adenomatous polyposis (4, 5) and sporadic colorectal cancers (3, 5–7). Apart from colorectal cancers, mutations in the APC gene also are associated with malignant brain tumors (Turcot’s syndrome (8)). Although APC is expressed constitutively within normal colon epithelium, little is known about how mutations of, or abnormal expression of, APC contribute to the development of colon cancer. Previous studies have indicated that the cellular levels of wild-type APC are critical to cytoskeletal integrity (9, 10), cellular adhesion (11), and Wingless/Wnt signaling (3, 12–17). Thus, understanding the mechanisms by which APC gene expression can be induced at the molecular level is critical.

The tumor suppressor gene p53 is also frequently mutated in colon cancer cells (3). The wild-type p53 protein is necessary for monitoring the G1 checkpoint, sensing DNA damage, assembling the DNA repair machinery, modulating gene amplification, or activating apoptosis to remove damaged cells (20). p53 activates transcription from specific DNA-binding sites and represses transcription in a binding site-independent manner. Treatment of cells with DNA-damaging agents induces nuclear accumulation of p53, which trans-activates cell cycle- and apoptosis-related genes (20). In previous studies, the transient expression of the wild-type APC gene into mammalian cells has been shown to cause cell cycle arrest (21) and apoptosis (22). However, whether APC expression in cells treated with DNA-damaging agents is increased and whether p53 plays any role in the expression of the APC are currently unknown. In the present investigation, we demonstrate that APC expression is, in fact, strongly induced after treatment of cells with DNA-damaging agents, such as the potent colon carcinogen N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) (for review, see Ref. 23). Furthermore, results indicated that the increased expression of the APC after MNNG treatment is dependent upon p53 expression.

Mammalian cells respond through a variety of signal transduction pathways after exposure to DNA-damaging agents, leading to specific programs of gene expression and alterations in the activity of proteins (24, 25) (for review, see Ref. 26). Among these effects, increased expression of tumor suppressor genes such as APC may be critical in controlling cellular transformation by delaying cell cycle progression or by inducing apoptosis. Our hypothesis is that exposure to DNA-damaging agents cause DNA damage, which eventually reach a threshold level at which DNA repair mechanisms fail, leading to the induction of apoptosis. We propose that APC, in collaboration with p53, is a critical component of cellular defense mechanisms involving DNA damage-induced cell cycle arrest and/or apoptosis.

* The abbreviations used are: APC, adenomatous polyposis coli; ActD, actinomycin D; CHX, cycloheximide; CMV, cytomegalovirus; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; DTT, dithiothreitol.

The paper is available on line at http://www.jbc.org
EXPERIMENTAL PROCEDURES

Maintenance and Treatment of Cells—Human cancer cell lines HCT-116 (colon), HeLa S3 (cervical), and K562 (erythroleukemia) were grown in McCoy’s 5A medium, Dulbecco’s modified Eagle’s medium (with high glucose), and RPMI medium, respectively, supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml penicillin and streptomycin (Life Technologies, Inc.). After cells reached to 70% confluence, fresh medium containing 0.5% FBS was added to each dish. Cells were further incubated for an additional 18 h and treated with DNA-alkylating agents for different periods.

Northern Blot Analysis—Total RNA was isolated by TRIZol reagent as described by the manufacturer (Life Technologies, Inc.). Fifty micrograms of total RNA were separated on 1% formaldehyde-agarose gel and transferred onto a Hybond-N* membrane (Amersham Corp.). The membrane was prehybridized for 6 h at 55 °C in 0.5 × sodium phosphate buffer (pH 7.2), 7% (w/v) SDS, 1 × EDTA, and 1% (w/v) bovine serum albumin and then hybridized with 32P-labeled APC probe (EcoRI fragment of APC-HFBC143 obtained from ATCC). Later the same membrane was reprobed with the 32P-labeled EcoRI fragment of the 18 S RNA probe for normalization of the total RNA loading and transfer efficiency. The membranes were exposed to x-ray films for detection of mRNA signals.

Western Blot Analysis—After treatment, cells were scraped off, pelleted, and washed with phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride. Cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.9, 2 mM MgCl₂, 2 mM DTT, 0.1% (v/v) Triton X-100, 0.1% (w/v) deoxycholate, 1 mM sodium metavanadate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin A. The cytosolic fraction was collected by centrifugation at 10,000 rpm for 5 min. Five-hundred micrograms of the cytosolic protein were immunoprecipitated with APC (N-15) polyclonal IgG (Santa Cruz Biotechnology), separated on a 2% amide gel electrophoresis. The proteins were electroblotted on an Immobilon-P membrane. The membrane was probed with the 32P-labeled antibody, signals were detected by the ECL Western blotting detection system (Amersham Corp.). The membrane was reprobed with the 32P-labeled EcoRI fragment of the 18 S RNA probe for normalization of the total RNA loading and transfer efficiency. The membranes were exposed to x-ray films for detection of mRNA signals.

Western Blot Analysis—After treatment, cells were scraped off, pelleted, and washed with phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride. Cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.9, 2 mM MgCl₂, 2 mM DTT, 0.1% (v/v) Triton X-100, 0.1% (w/v) deoxycholate, 1 mM sodium metavanadate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin A. The cytosolic fraction was collected by centrifugation at 10,000 rpm for 5 min. Five-hundred micrograms of the cytosolic protein were immunoprecipitated with APC (N-15) polyclonal IgG (Santa Cruz Biotechnology), separated on a 2% amide gel electrophoresis. The proteins were electroblotted on an Immobilon-P membrane. The membrane was probed with the 32P-labeled antibody, signals were detected by the ECL Western blotting detection system (Amersham Corp.). The membrane was reprobed with the 32P-labeled EcoRI fragment of the 18 S RNA probe for normalization of the total RNA loading and transfer efficiency. The membranes were exposed to x-ray films for detection of mRNA signals.

RESULTS AND DISCUSSION

Induction of Expression of APC by DNA-damaging Agents—In the past few years, it has been clearly demonstrated that the tumor suppressor APC plays an important role in the development of colorectal cancer (3). However, the biological function(s) of APC is poorly understood. To gain insight into the biological function(s) of APC, it is critical to understand the molecular mechanisms of the APC gene expression. To examine the possibility of whether APC is inducible, an HCT-116 human colon cancer cell line, which expresses wild-type APC (18), was exposed to DNA-damaging agents such as MNNG (50 μM), dimethylhydrazine (DMH, 250 μM), 3-morpholinosydnonimine (SIN-1, 100 μM), carboplatin (50 μM), melphalan (50 μM), mecloatrexine (1 μM), and ultraviolet (UV) light (5 J/m²). The APC mRNA levels were increased after treating cells with all of these DNA-damaging agents, indicating the global nature of this response (Fig. 1A). The maximum increase in the APC mRNA level was observed with MNNG, which was used in subsequent experiments. A dose-dependent effect of treatment with MNNG on the induction of APC mRNA is shown in Fig. 1B. Continuous exposure of HCT-116 cells to MNNG (50 μM) induced an 8–12-fold increase in the levels of both APC mRNA and protein in a time-dependent manner (Fig. 1, C and D, respectively).

MNNG Induces Transcriptional Regulation of APC in HCT-116 Cells—To investigate the mechanisms regulating transcription of APC, cells were treated simultaneously with MNNG and actinomycin D (ActD) or with cycloheximide (CHX) for 15 h (Fig. 2A) to block transcription and new protein synthesis, respectively. Whereas treatment with ActD abolished MNNG’s induction of APC mRNA expression, that treatment with CHX served to induce APC implicates a negative post-transcriptional regulatory mechanism involving a labile factor. To investigate the effect of MNNG treatment on the stability of the APC mRNA, the half-life (t₁/₂) of transcribed APC mRNA was determined in control versus MNNG-treated cells by treatment with ActD (1 μg/ml) or buffer for different periods to block further transcription. The kinetics of APC mRNA degradation (t₁/₂ of 125–150 min) with or without MNNG indicated that the increased APC expression was not due to a post-transcriptional mechanism (Fig. 2B). Nuclear run-on assays showed that nascent APC mRNA synthesis was higher in nuclei isolated from

FIG. 1. Expression of APC mRNA in the HCT-116 human colon cancer cell line after treatment with DNA-damaging agents. The HCT-116 cells were treated with various agents in culture for 15 h (A) or with different concentrations of MNNG for 15 h (B). The stock solution of MNNG was prepared in dimethyl sulfoxide, which was diluted in the medium giving a final concentration of 0.1% (v/v). C and D show the effect of 50 μM MNNG treatment for different periods on the APC mRNA and protein levels, respectively. The amount of APC mRNA was measured by Northern blot analysis and normalized to 18 S RNA. The APC mRNA signals were quantified densitometrically and presented as relative units of APC mRNA/18 S RNA ratio. Data of A, C, and D are representative of two independent experiments, and data of B are mean ± S.D. of three experiments.
MNNG-treated versus control cells, whereas the rate of transcription for 18 S RNA remained unchanged (Fig. 2C). Collectively, these data establish that transcriptional rather than post-transcriptional mechanism(s) are involved in the regulation of APC after DNA alkylation damage.

Endogenous p53 Expression Is Required for Induction of APC mRNA Levels by MNNG—The expression of the tumor suppressor p53 is induced by a wide variety of DNA-damaging agents and has been proposed to play a necessary role in controlling transcription of the critical genes involved in cell cycle arrest and DNA replication (20). Since p53 is also involved in cell cycle arrest (21) and we have discovered that APC expression is transcriptionally induced after DNA damage, we next examined a role for p53 in APC expression. Thus, if p53 is required for DNA damage-induced expression of APC, then cell lines defective in wild-type functional p53 should fail to up-regulate APC. Three cell lines with different p53 expression were selected: (i) the HCT-116 cell line expresses wild-type p53 (29); (ii) the HeLa S3 cell line expresses barely detectable levels of p53 protein (30); (iii) the erythroleukemic K562 cell line is negative for the p53 gene and fails to express p53 protein (31). Analysis of RNA from these cell lines treated with different concentrations of MNNG for 15 h (Fig. 3) indicated a tight correlation between the p53 status and the induction of steady-state levels of APC mRNA after MNNG treatment. The HCT-116 cell line showed a dose-dependent increase in the APC mRNA level (Fig. 3A), whereas HeLa cells showed only a slight (i.e., <2-fold) increase in expression at 25–50 μM MNNG, and the basal level of APC expression in p53-negative K562 cells was unaffected by DNA damage (Fig. 3A). Since exposure of mammalian cells to DNA-damaging agents increases cellular levels of p53 protein (20), we examined whether the increase in APC mRNA levels in HCT-116 cells after MNNG treatment was correlated with a simultaneous increase in p53 protein levels. The p53 protein levels were increased significantly in HCT-116 cells after treatment with MNNG (50 μM) but were not altered in HeLa cells (Fig. 3B). These results suggest that APC expression is sensitive to, and may require, increased levels of p53.

After DNA damage, increased levels of cellular p53 protein transcriptionally activate genes required for cell cycle arrest and apoptosis (20). For example, p53 up-regulates expression of the WAF1/Cip1 gene, which is a known inhibitor of cyclin/Cdk function (32, 33). The decreased cyclin/Cdk activity leads to hypophosphorylation of pRb, resulting in cell cycle arrest at the G1 phase (20). In the present studies, WAF1/Cip1 protein levels remained unchanged after MNNG treatment of p53-expressing HCT-116 cells, whereas pRb levels significantly decreased (data not shown). These results suggest that p53-mediated MNNG-induced signaling does not involve WAF1/Cip1 or pRb. Since we observed a consistent requirement for p53 in APC up-regulation, it appears that a p53-mediated signaling pathway is required for APC regulation in response to DNA dam-

![Figure 2. Transcriptional up-regulation of APC in HCT-116 cells after MNNG treatment for 15 h. A, effects of ActD and CHX on MNNG-induced APC mRNA synthesis. To examine the effect of MNNG on APC mRNA synthesis, HCT-116 cells were treated with 1 μg/ml ActD or 5 μg/ml CHX in the culture medium along with 90 μM MNNG. After 15 h of treatment, total RNA was isolated. B, kinetic analysis of MNNG-induced APC mRNA stability. To determine APC mRNA stability, after 15 h of MNNG treatment control and treated cells were exposed to 1 μg/ml ActD, and cells were harvested after different additional incubation periods for isolation of total RNA. The APC mRNA signals determined by Northern blotting were normalized to 18 S RNA signals and plotted on a logarithmic scale. C, nuclear run-on assay. To examine the induced rate of APC mRNA synthesis, a nuclear run-on transcription assay was performed with isolated nuclei from HCT-116 cells (either control or treated with 50 μM MNNG for 15 h). *P-Labeled RNA were isolated and hybridized with membrane-bound APC and 18 S cDNA probes and poly(dIdC) as a nonspecific DNA control. Data presented are representative of four (A) and two (B and C) independent experiments.](image)

![Figure 3. Comparison of the APC mRNA and p53 protein levels in various cell lines after MNNG treatment. Cells were treated with different concentrations of MNNG for 15 h in duplicate for total RNA and nuclear extract preparations. A, relative amounts of APC mRNA. B, Western blot analysis of p53. The inset shows a photograph of a p53 Western blot of control (C) and 50 μM MNNG (M)-treated cells. The p53 protein bands were quantified, and the ratio of expression in treated versus untreated cultures are shown in the main panel. Data are representative of three independent experiments.](image)
Recent studies have indicated that APC is an important component of the Wnt signaling pathway (3, 14). In the absence of Wnt signals, APC interacts with and is phosphorylated by glycyogen synthase kinase-3β, which regulates cellular levels of β-catenin and inhibits signaling by the β-catenin-Tcf/Lef complexes (15, 16). These studies have suggested that in the absence of wild-type APC, β-catenin-Tcf/Lef complexes are stabilized and translocated into the nucleus, where they may activate transcription of the target genes, which might be involved in the development of colorectal cancer (15, 16) as well as in melanoma (17). Besides being a negative regulator of β-catenin signaling, APC has also been implicated in the migration of intestinal epithelial cells from the crypt up to the villi, where these cells undergo apoptosis. This physiological process is necessary to maintain the normal functioning of the intestinal cells (for review, see Ref. 28). Previous studies have suggested that free β-catenin blocks the APC-mediated cell migration. Thus increased levels of the wild-type APC and low levels of β-catenin can independently regulate gene expression by controlling the levels of β-catenin-Tcf/Lef complexes and similarly affect also the migration of epithelial cells. How DNA damage-induced APC levels play a role in these processes is currently unknown. Thus, our findings support the idea that DNA-damaging agents can induce transcription of the APC gene in a p53-dependent manner, which might perhaps inhibit β-catenin-Tcf/Lef complex signaling and also might play a role in cell migration and apoptosis. Nevertheless, the dependence of APC expression on p53 may be highly physiologically relevant in that a p53-APC connection may help to identify a functional role for the APC. Obviously, when the vital cell processes are dysfunctional, as in cells containing mutant APC and/or p53, then increased genetic instability results and may lead to the development of cancer (for reviews, see Refs. 3 and 34). Increased APC levels may overcome this process, either by cell cycle arrest and DNA damage repair or by inducing apoptosis.

Acknowledgments—We thank Drs. W. Stratford May, Samuel H. Wilson, Brian Davis, Stephen R. Lloyd, Bennett Van Houten, David Konkel, and Phyllis Strausser for helpful suggestions and critical reading of the manuscript. We are grateful to Dr. W. Stratford May, Director of the Sealy Center for Oncology and Hematology, for support and encouragement during the course of this investigation, to Drs. S. Swaminathan and Lei Xiao for plasmids, and to Sanjay Awasthi for providing chemotherapeutic drugs.

REFERENCES

1. Nagao, M., and Sugimura, T. (1993) Mutat. Res. 290, 43–51

2. Giovannucci, E., and Willett, W. C. (1994) Ann. N. Y. Acad. Sci. 749, 452–455

3. Kinzler, K. W., and Vogelstein, B. (1996) Cell 87, 159–170

4. Ichii, S., Horii, A., Nakatani, S., Furuyama, J., Utsunomiya, J., and Nakamura, Y. (1992) Hum. Mol. Genet. 1, 387–390

5. Powell, S. M., Zilz, N., Beazer-Barclay, Y., Bryan, T. M., Hamilton, S. R., Thibodeau, S. N., Vogelstein, B., and Kinzler, K. W. (1992) Nature 359, 205–207

6. Miyoshi, Y., Nagase, H., Ando, H., Horii, A., Ichii, S., Nakatani, S., Aoki, T., Miki, Y., Mori, T., and Nakamura, Y. (1992) Hum. Mol. Genet. 1, 229–233

7. Tao, J., and Shibata, D. (1994) Am. J. Pathol. 145, 531–534

8. Hamilton, S. R., Liu, B., Parsons, R. H., Papadopoulos, N., Jen, J., Powell, S. M., Krush, A. J., Berk, T., Cohen, Z., Tetu, B., Burger, P., Wood, P. A., Taqi, F., Booker, S. V., Peterson, G. M., O’Geffhausen, G. H. J., A. Tarsomettes, M. A., Giardello, F. M., Vogelstein, B., and Kinzler, K. W. (1995) N. Engl. J. Med. 332, 829–847

9. Munemitsu, S., Souza, B., Muller, O. Albert, I., Rubinfeld, B., and Polakis, P. (1994) Cancer Res. 54, 3676–3681

10. Smith, K. J., Levy, D. B., Maupin, P., Pollard, T. D., Vogelstein, B., and Kinzler, K. W. (1994) Cancer Res. 54, 3672–3675

11. Barth, A. I. M., Pollack, A. L., Altschuler, Y., Mostov, K. E., and Wilson, W. J. (1997) J. Cell Biol. 136, 693–706

12. Rubinfeld, B., Souza, B., Albert, I., Muller, O., Chamberlain, S. H., Masiarz, F. R., Munemitsu, S., and Polakis, P. (1993) Science 262, 1731–1734

13. Su, L. K., Vogelstein, B., and Kinzler, K. W. (1993) Science 262, 1734–1737

14. Ruinhein, B., Albert, I., Polakis, P. (1996) Science 272, 1023–1026

15. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., and Vogelstein, B. (1997) Science 275, 1784–1787

16. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Cleavers, H. Vogelstein, B., and Kinzler, K. W. (1997) Science 275, 1787–1790

17. Ruinhein, B., Rubinfen, P., El-Gamil, M., Albert, I., Polakis, P., and Polakis, P. (1997) Science 275, 1790–1792

18. Groden, J., Joslyn, G., Samowiz, W., Jones, D., Bhattacharyya, N., Spiro, L., Thiveria, A., Robertson, M., Eagan, S., Muth, M., and White, R. (1995) Cancer Res. 55, 1531–1534

19. Hargest, R., and Williamson, R. (1995) Gut 37, 826–829

20. Levine, A. J. (1997) Cell 88, 323–331

21. Konkel, J., Matsunaga, A., Konoda, T., Bhatiacharjya, R. N., Miyashiro, I., Toyoshima, K., and Akiya, T. (1995) EMBO J. 14, 5618–5625

22. Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 7950–7954

23. Umemi, I. (1995) Bioch. Rev. 70, 107–160

24. Narayan, S., Beard, W. A., and Wilson, S. H. (1995) Biochemistry 34, 73–80

25. Narayan, S., Be, F., and Wilson, S. H. (1996) J. Biol. Chem. 271, 15809–15813

26. Sato, K., and Shibata, D. (1994) Science 265, 4717–4719

27. Smith, K. J., Johnson, K. A., Bryan, T. M., Hill, D. E., Markowitz, S., Wilson, S. M., Krush, A. J., Berk, T., Cohen, Z., Tetu, B., Burger, P., Wood, P. A., Taqi, F., Booker, S. V., Peterson, G. M., O’Geffhausen, G. H. J., A. Tarsomettes, M. A., Giardello, F. M., Vogelstein, B., and Kinzler, K. W. (1992) Science 251, 1731–1734

28. Peifer, M. (1997) Science 275, 1752–1753

29. Waldren, T., Kinzler, K. W., and Vogelstein, B. (1995) Cancer Res. 55, 5187–5190

30. Scheffner, M., Werness, B. A., Huihrest, J. M., Levine, A. J., and Howley, P. M. (1996) Cell 63, 1129–1136

31. Soldo, S., Blandino, G., Scardigli, B., Reom, S., Marchetti, A., Rizzio, M. G., Bosni, G., Chimino, L., Crescenti, M., and Sacchi, A. (1996) J. Cell Biol. 134, 193–204

32. Harper, J. W., Adam, G. R., Wei, N., Keyomarski, K., and Elledge, S. J. (1993) Cell 75, 817–825

33. Cheng, K. C., and Loeb, L. A. (1997) Curr. Top. Microbiol. Immunol. 221, 5–18