Anti-Cancer Drugs 1997, 8, pp. 56–61

Activation of the retinoic acid receptor β gene by 5-aza-2′-deoxycytidine in human DLD-1 colon carcinoma cells

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We previously reported that 5-aza-2′-deoxycytidine (5-Aza-CdR) in combination with all-*trans* retinoic acid (ATRA) produced a synergistic antineoplastic effect on DLD-1 colon carcinoma cells. We also observed that 5-Aza-CdR, a potent inhibitor of DNA methylation, increased the expression of retinoic acid receptor (RAR)-β. Methylation of cytosine in the promoter–first exon region of genes has been reported to silence their expression. In an attempt to clarify the mechanism responsible for the activation of the RAR-β gene by 5-Aza-CdR in DLD-1 colon carcinoma cells, we investigated its methylation state by Southern blotting. Our results indicate that DNA hypermethylation of the RAR-β gene, a putative tumor suppressor gene, may be the mechanism of silencing its expression in these tumor cells. We also reported that a different schedule of 5-Aza-CdR and ATRA produced a synergistic antineoplastic effect on the colon carcinoma cells.

Key words: 5-Aza-2′-deoxycytidine, colon carcinoma, DNA methylation, retinoic acid, retinoic acid receptor β.

Introduction

Since metastatic colon cancer responds poorly to conventional chemotherapy,[1] there is an urgent need to develop more effective therapy for this disease. An interesting approach is to use differentiation therapy with an agent such as retinoic acid which has been demonstrated to induce *in vitro* differentiation and growth inhibition of different types of neoplastic cells.[2,3] The action of all-*trans* retinoic acid (ATRA), a metabolite of vitamin A, is due to its binding to nuclear retinoic acid receptors (RAR) which are ligand-activated transcription factors.[4,5] The RARs bind to a retinoic acid responsive element in the promoter region of specific genes to produce an activation or suppression of gene expression.[6] Three types of RAR have been identified: RAR-α, RAR-β and RAR-γ; their DNA sequences being very homologous in the ligand and DNA-binding regions.[7–9]

RAR-β has been proposed to be a tumor suppressor gene.[10–12] This hypothesis is supported by several lines of evidence. The lack of expression of RAR-β has been observed in several different types of tumor cells including lung carcinoma,[13,14] squamous cells carcinoma of the pharynx,[15] ovarian cancer[16] and breast cancer cells.[17] Gene transfer of RAR-β cDNA into tumor cells that do not express this gene resulted in a suppression of tumor growth.[10,18] The specific loss of RAR-β function may be an important event in the progression to a neoplastic phenotype.

Several recent reports have shown that hypermethylation of normally unmethylated CpG islands in the promoter region of tumor suppressor genes, including p16,[19–21] VHL,[22] Rb[23] and p15,[24] correlates with loss of transcription. These findings suggest an alternative mechanism for tumor suppressor inactivation which differs from gene inactivation produced by chromosomal deletion or mutation.

Since the loss of RAR-β expression in tumor cells confers resistance to retinoic acid treatment,[25] an interesting agent to use in combination with ATRA to overcome this problem is 5-aza-2′-deoxycytidine (5-Aza-CdR). This cytosine analog is a potent inhibitor of DNA methylation which can lead to gene activation and induction of cellular differentiation.[26] 5-Aza-CdR was demonstrated to be an active drug in patients with acute leukemia.[27,28] In addition, 5-Aza-CdR has been reported to activate the expression of several different tumor suppressor genes.[22,24,29]

In our previous *in vitro* study, we demonstrated a synergistic antineoplastic interaction between ATRA and 5-Aza-CdR on human DLD-1 colon carcinoma cells.[30] One objective of this study was to determine

This work was supported by grants from the Cancer Research Society and the National Cancer Institute of Canada.

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if another schedule for this combination could produce a similar interaction. The other objective of this study was to understand the molecular mechanism of this interaction by which 5-Aza-CdR activates the expression of RAR-β in DLD-1 cells making them more sensitive to ATRA. Southern blot analysis showed that hypermethylation of the RAR-β gene may be a mechanism of silencing this gene in these cells and the demethylation by 5-Aza-CdR can produce its activation.

Materials and methods

Materials

Minimal essential medium (MEM), non-essential amino acids and 0.25% trypsin–1 mM EDTA were obtained from Canadian Life Technologies (Burlington, Ontario, Canada). Fetal calf serum (FCS) was obtained from Wisent (Montreal, Quebec, Canada). ATRA was obtained from Sigma (St Louis, MO), dissolved in absolute ethanol, protected from light and stored at −20°C. 5-Aza-CdR was obtained from Mack (Illertissen, Germany), dissolved in 0.45% NaCl containing 10 mM sodium phosphate (pH 6.8) and stored at −70°C. [α-^{32}P]dCTP (3000 Ci/mmol) was obtained from ICN Canada (Mississauga, Ontario, Canada).

Cell culture

Human DLD-1 colon adenocarcinoma cell line was obtained from the American Type Tissue Culture Collection (Rockville, MD). The tumor cell line was maintained in culture in MEM containing non-essential amino acids and 10% heat-inactivated FCS in a 5% CO_2 incubator at 37°C. The doubling time for the DLD-1 cell line was between 24 and 28 h. For subculture, the cells were placed in 0.25% trypsin–1 mM EDTA to obtain the cell suspension.

Clonogenic assay

A 5 ml aliquot (150 cells) was suspended in MEM containing 10% heat-inactivated FCS and placed in a 25 cm^2 tissue culture flask and incubated for 48 h at 37°C in a 5% CO_2 incubator. For sequential treatment, the indicated concentration of 5-Aza-CdR was added on day 0 and ATRA was added on day 3; both drugs were removed on day 5. The exposure times for 5-Aza-CdR and ATRA were 120 and 48 h, respectively. After an incubation at 37°C in 5% CO_2 for 7 days, the colonies (more than 500 cells) were rinsed, stained with 0.5% methylene blue in 50% methanol and counted. The loss of clonogenicity values is expressed as percentage of survival relative to control. The cloning efficiency of the control cells was in the range of 85–90%.

Northern blot analysis

Total RNA was isolated with the Ultraspec-II RNA isolation system (Biotecx, Houston, TX). Briefly, after homogenization in the denaturing solution of guanidine and urea, a specific RNA binding resin was used to purify the total RNA. The pure RNA was eluted from resin and poly(A)$^+$ RNA was prepared by oligo(dT)–cellulose chromatography (5 Prime–3 Prime, Boulder, CO). Poly(A)$^+$ RNA (2.5 μg) was size fractionated in formaldehyde agarose gel. The mRNA was transferred to a Hybond-N nylon membrane (Amersham, Oakville, Ontario, Canada). The membrane was baked at 80°C for 90 min and fixed under UV light. The human RAR-β probe used was the 1.4 kb *Sac*I–*Bam*HI DNA insert from plasmid pSG5 (gift from Dr J White, McGill University) and was labeled with [α-^{32}P]dCTP by the random prime reaction. The blot was hybridized with this probe (3×10^6 c.p.m./ml) and exposed to X-ray film for autoradiographic analysis. As control for the relative amount of mRNA, the blot was hybridized to a 983 bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA probe synthesized by PCR. The densitometric analysis was performed using the Pharmacia-LKB Ultroscan XL Enhanced Laser Densitometer.

Southern blot analysis

Genomic DNA (5 μg) was digested overnight with *Xba*I (10 units/μg), *Hpa*II (15 units/μg) or *Msp*I (15 units/μg). The restriction fragments were separated by electrophoresis through 1% agarose gel, transferred to a Hybond-N nylon membrane and hybridized with a PCR DNA probe labeled with ^{32}P (2×10^6 c.p.m./ml). The DNA probe was prepared by the PCR amplification of a 227 bp fragment from the A region (exon 3) of RAR-β_2 with the following primers: sense, 5'-AGA GTT TGA TGG AGT TGG GTG GAC-3' and antisense, 5'-CAT TCG GTT TGG GTC AAT CCA CTG-3'.[31] After hybridization, nylon filters were washed and exposed to X-ray film at −70°C for 48 h for autoradiography.

Results

Evaluation of antineoplastic action of 5-Aza-CdR and ATRA

The reduction of colony formation produced by 5-Aza-CdR or ATRA alone and in combination on the DLD-1 colon carcinoma cell line is shown in Table 1. A sequential treatment was used in which 5-Aza-CdR was added on day 0 and ATRA was added on day 3. Both drugs were removed from the medium on day 5. As evaluated by a colony assay, under these experimental conditions 5-Aza-CdR (0.8 μM) alone produced 53.6% loss of clonogenicity, whereas ATRA (1.0 μM) alone showed less than 1% loss of clonogenicity. The combination of these agents produced 77.7% loss of clonogenicity, a clearly synergistic interaction as described by Momparler.[34]

Analysis of DNA methylation status of RAR-β by Southern blotting

Figure 1 shows a methylation-sensitive restriction map of the 5′ promoter region and exon 3 of the RAR-β_2 gene. Exon 3 contains the 5′ untranslated and the A regions of this gene. There are several sites for the methylation-sensitive restriction enzyme *Hpa*II in this region. This enzyme cuts C/CGG sites only if the cytosine residue is not methylated. The bottom part of the figure shows the predicted size of the DNA fragments from this region. The 227 bp DNA probe used to detect the presence of these fragments on a Southern blot was from 3′ end of exon 3.

Figure 2 shows the Southern blot of genomic DNA digested with *Xba*I and/or *Hpa*II from DLD-1 colon

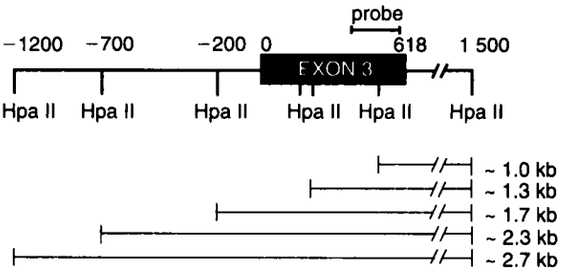

Figure 1. Restriction map of the promoter–exon region of the RAR-β gene for the methylation-sensitive *Hpa*II enzyme. Exon 3 contains the 5′ untranslated region and the coding A region of RAR-β_2.[31] The 227 bp DNA probe was generated by PCR and was from nucleotide positions 377 to 603 of exon 3. The bottom part of the figure shows the expected fragment sizes recognized by this probe.

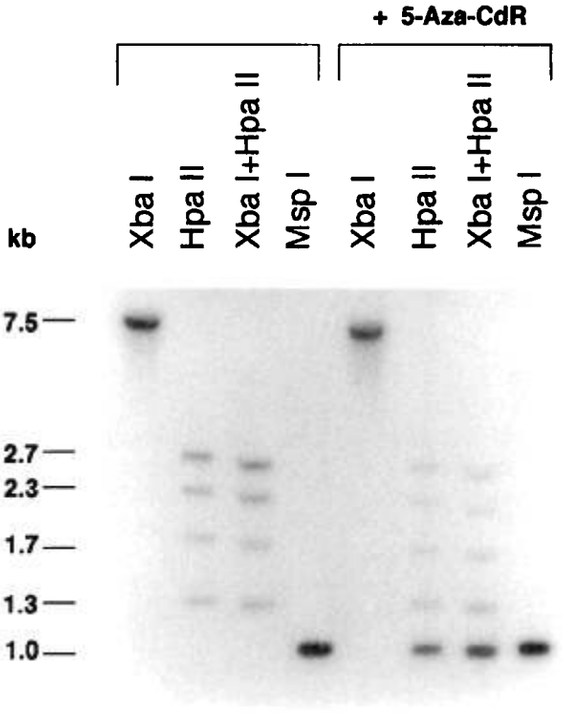

Figure 2. Evaluation of the methylation status of the RAR-β gene by Southern blot analysis of DLD-1 colon carcinoma cells before and after treatment with 0.5 μM 5-Aza-CdR for 3 days. Genomic DNA (5 μg) was digested with *Xba*I, *Hpa*II, *Xba*I + *Hpa*II or *Msp*I before 5-Aza-CdR (lanes 1, 2, 3 and 4, respectively) and after 5-Aza-CdR (lanes 5, 6, 7 and 8, respectively). The blot was hybridized with a 227 bp DNA probe that was labeled with ^{32}P (see Figure 1). *Msp*I cuts a the same site as *Hpa*II, but is not inhibited by methylation.

Table 1. Effect of sequential treatment of 5-Aza-CdR and ATRA on the loss of clonogenicity by DLD-1 colon carcinoma cells[a]

5-Aza-CdR (μM)	ATRA (μM)	Loss of clonogenicity (%)	Interaction[b]
0.4	0	34.3 ± 0.5[c]	
0.8	0	53.6 ± 1.2	
0	1	< 1	
0.4	1	42.8 ± 2.4	synergistic
0.8	1	77.7 ± 1.0	synergistic

[a]5-Aza-CdR was added on day 0 and ATRA added on day 3. Both drugs were removed on day 5.
[b]Determined as described by Momparler.[34]
[c]Mean ± SE (n = 4).

carcinoma cells before (lanes 1–4) and after (lanes 5–8) treatment with 0.5 μM 5-Aza-CdR for 3 days. For the untreated cells, we detected four bands of 1.3, 1.7, 2.3 and 2.7 kb (lanes 2 and 3) after digestion with *Hpa*II alone and with *Xba*I plus *Hpa*II, respectively. After treatment with 5-Aza-CdR, these bands became very faint and we detected a major band at about 1.0 kb (lanes 6 and 7). Digestion of the DNA by *Msp*I from both untreated and 5-Aza-CdR-treated cells (lanes 4 and 8) produced only one major band at about 1.0 kb. *Msp*I, which was used as an internal control, cuts at the identical site as *Hpa*II, even if the cytosine residue is methylated. A concordant methylation pattern was obtained using the methylation-sensitive enzyme *Hha*I (data not shown).

Evaluation of RAR-β mRNA expression by Northern blot analysis

Figure 3 indicates that mRNA for RAR-β was not expressed in untreated DLD-1 colon carcinoma cells by Northern blot analysis using a probe of the RAR-β gene labeled with ^{32}P. Treatment of these tumor cells with 0.5 μM of 5-Aza-CdR for 5 days increased the mRNA expression of RAR-β as shown by the presence of a band at about 3.1 kb. As a reference standard for an equivalent amount of mRNA loaded in each lane, we used the GAPDH DNA probe which showed bands of similar intensity for each group.

Discussion

The use of agents that induce differentiation merits investigation since advanced metastatic colon carcinoma responds poorly to conventional chemotherapy.[1] In this report we have investigated the *in vitro* antineoplastic action of sequential treatment of 5-Aza-CdR and ATRA on human DLD-1 colon carcinoma cells. Clonogenicity is an important characteristic of tumor cells as it is an index of the proliferative potential of these cells. We performed a colony assay to determine if the sequential treatment of DLD-1 colon carcinoma cells with these drugs in combination would produce a synergistic reduction in clonogenicity. The results clearly showed that the

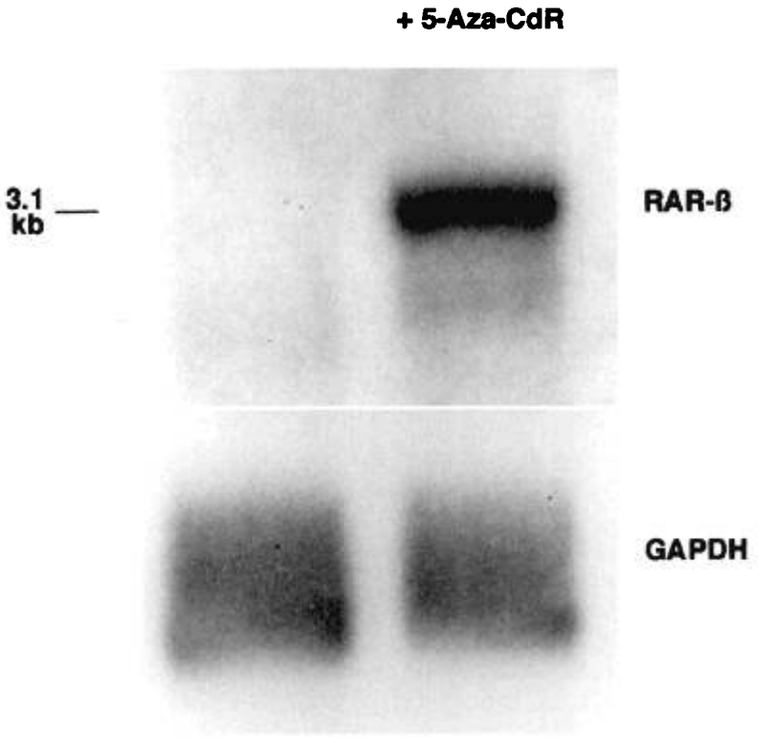

Figure 3. Activation of the expression of RAR-β in DLD-1 colon carcinoma cells by treatment with 0.5 μM 5-Aza-CdR for 5 days. Northern blot analysis was performed on 2.5 μg poly(A)$^+$ RNA isolated before (lane 1) and after (lane 2) treatment with 5-Aza-CdR. The blot was hybridized with DNA probes labeled with ^{32}P for RAR-β and GAPDH as described in Materials and methods.

interaction between these two agents in sequential treatment was synergistic (Table 1). These results confirm our previous work where we used a different schedule of simultaneous drug exposure.[30]

The inactivation of tumor suppressor genes can be implicated in the progression of neoplasia. Historically, the initial observations showed that these genes could be inactivated by chromosomal deletion and mutation. Recent reports indicate that DNA methylation in the promoter region of tumor suppressor genes is an additional mechanism to silence the expression of these genes.[19–21]

Observations from several investigators suggest that RAR-β can act as a tumor suppressor gene.[10–12] RAR-β is not expressed in many different types of tumors.[13–17] Our results suggest that the silencing of the expression of this gene in DLD-1 colon cancer cells occurs by DNA methylation. We have observed by Northern blot analysis that RAR-β is not expressed in DLD-1 colon carcinoma cells and that treatment with the DNA methylation inhibitor, 5-Aza-CdR, activated the expression of this gene (Figure 3).

Southern blot analysis of the methylation sites in the promoter region of the RAR-β (Figure 2) before and after treatment with 5-Aza-CdR showed that this analog demethylated a site in exon 3 which may play a key role in the control of the expression of this gene. Exon 3 contains the initiation codon ATG for the A region of the RAR-β protein.[31] The methylation-sensitive enzyme *Hpa*II was used in this analysis. This enzyme cuts the C/CGG site only if the cytosine residue is not methylated. Untreated colon carcinoma cells showed 1.3, 1.7, 2.3 and 2.7 kb bands after digestion with *Hpa*II, suggesting that the restriction sites for this enzyme in the intron 3 promoter region of RAR-β showed variable methylation (Figure 2). Treatment of the cells with 5-Aza-CdR produced one major band at about 1.0 kb after digestion with *Hpa*II. This suggests from the *Hpa*II restriction map (Figure 1) that a major demethylation site in exon 3 is involved in the control of expression of RAR-β. Digestion of the DNA with the enzyme *Msp*I, which cuts at the identical site as *Hpa*II even if the cytosine residue is methylated, gave identical results showing a single band at about 1.0 kb. It is interesting to note that key *Hpa*II sites were identified in the region of the initiator codon region of the tumor suppressor genes VHL and p15.[22,24] The very faint bands over 1 kb observed in the Southern blot from 5-Aza-CdR-treated cells probably represent a small fraction of the cell population in which remethylation of some *Hpa*II sites took place after drug decomposition.[32]

DNA methylation in the promoter region may inhibit gene expression by indirectly hindering the binding of transcription factors.[33] In addition to chromosomal deletion and mutation, aberrant DNA hypermethylation may play an important role in the inactivation of tumor suppressor genes.

Our results suggest that hypermethylation was responsible for the loss of the RAR-β gene expression and that the activation of this putative tumor suppressor gene by 5-Aza-CdR in colon carcinoma cells indicates a possible role of this analog in cancer therapy. In addition, the use of retinoids in combination with 5-Aza-CdR has the potential to enhance the antitumor activity of this interesting analog.

Acknowledgments

SC was supported by a studentship from FCAR.

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(Received 8 October 1996; accepted 31 October 1996)