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DEMETHYLATION OF THE TYPE I PROCOLLAGEN GENES IN TRANSFORMED FIBROBLASTS TREATED WITH 5-AZACYTIDINE

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SUMMARY. Transformation of the human embryonic lung fibroblast line, WI-38, with simian virus 40 (SV40) results in inactivation of the type I procollagen genes. No type I collagen or procollagen mRNA is detected in these transformed cells, as determined by polyacrylamide gel electrophoresis. Analysis of the methylation patterns of these genes showed the type I procollagen genes to be hypermethylated at certain cytosine residues in the transformed cells. However, several of the cytosine residues were methylated in the normal cells where these genes are expressed. These methylation patterns can be altered by treatment of the cells with 5-azacytidine or 5-azadeoxycytidine, but without a resultant activation of the type I procollagen genes. These results show that demethylation alone is not sufficient for gene activation, but that other signals are also required.

INTRODUCTION. Several studies have implicated methylation of cytosine residues in the regulation of gene expression (for review see ref. 1). Usually inactive genes are hypermethylated when compared to their active counterparts. In normal eukaryotic cells approximately 3-5% of the cytosines are modified to 5-methylcytosine (2), with about 90% occurring in the dinucleotide sequence CpG. This cytosine methylation is a post-replicative event, with the methyl group being donated by S-adenosyl-methionine. When cells are treated with the unstable cytidine analogue 5-azacytidine or with its deoxy derivative, the DNA methyltransferase is unable to methylate the nitrogen atom at the 5' position of the pyrimidine ring (3). This situation results in loss of enzyme and profoundly decreased DNA methylation (4) and this is very often accompanied by altered gene expression. In the first studies 5-azacytidine was shown to

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induce conversion of mouse C3H/10T½ fibroblasts to myotubes (5,6) as well as to chondrocytes and adipocytes. Several studies have also shown that inactive genes (7,8,9) or retroviruses (10) can be activated by 5-azacytidine treatment.

We have previously shown that a line of SV40 transformed WI-38 human embryonic lung fibroblasts (SVWI-38) does not produce type I collagen, and that its α1(I) and α2 procollagen genes are hypermethylated when compared to normal WI-38 fibroblasts (11). In this study we show that the type I procollagen genes in the SVWI-38 cells can be demethylated by 5-azacytidine treatment, but that no type I collagen is produced. Also, in spite of inducing gross demethylation of the DNA, no phenotypic conversions occur such as those observed in the case of mouse C3H/10T½ fibroblasts.

METHODS

Cell Culture. WI-38 (ATCC CL75) and SVWI-38 cells were cultured in Eagle's basal medium containing 10% heat-inactivated fetal calf serum. Treatment with 5-azacytidine (AzaC) or 5-azadeoxycytidine (AzaCdR) was either for 18 hours during log phase, or for 8 hours during the S phase, after synchronization with a double thymidine block (20).

Collagen synthesis. Cells were labelled with 10 μCi/ml [^{3}H]-proline (Amersham) for 24 hours in the presence of 50 μg/ml ascorbate and 50 μg/ml β-aminopropionitrile. The medium was removed and centrifuged at 10000 x g for 10 minutes to remove cellular debris. The supernatant was adjusted to 0.5 M with respect to acetic acid and digested with 100 μg/ml pepsin for 16 hours at 15°C. After neutralization and extensive dialysis, the samples were lyophilized and analyzed on 20 cm long 7% polyacrylamide gels (12).

DNA isolation and hybridization. Cells from 150 cm^2 flasks were lysed in 2 x SSC (SSC - 0.15 M NaCl, 0.015 M Na citrate), 1% SDS and digested with 100 μg/ml proteinase K (Merck) for 1 hour at 50°C. The samples were then deproteinized by repeated phenol extractions as previously described (11). After digestion with the indicated restriction endonucleases (New England Biolabs, USA, or Boehringer), the DNA was electrophoresed on 1% horizontal agarose gels and transferred to nitrocellulose as described by Southern (13). DNA was digested with 2 units of enzyme/μg DNA for 2 hours at 37°C. Completeness of digestion was monitored by addition of λ-DNA to some of the samples. The nitrocellulose sheets were hybridized to ^{32}P-labelled chicken type I procollagen clones (14,15) pCg 45 (α2) and pCg 54 (α1(I)), as previously described (11). These clones were generously supplied by Dr. Helga Boedtker (Harvard University, USA). All recombinant DNA experiments were done in compliance with NIH guidelines.

Extracellular matrix analysis. Cells were grown to confluence in 30 mm dishes in the presence of 50 µg/ml ascorbate. Three days prior to harvesting, [^{3}H]-proline was added at 1 µCi/ml. The cells were lysed with 0.05% SDS, and the matrix left behind on the dish was washed 3 times with phosphate-buffered saline and 3 times with 96% ethanol. After drying, the matrix was digested with 100 µg/ml trypsin in 10 mM Tris pH 7.6, 10 mM $CaCl_2$ for 20 hours at 37°C. The digested material was removed for scintillation counting and the residual matrix digested with 100 µg/ml collagenase (Worthington CSLPA) in the above buffer containing 2.5 mM n-ethylmaleimide in order to inhibit non-specific proteases (14).

RESULTS

Analysis of the extracellular matrix produced by WI-38 and SVWI-38 fibroblasts showed that the transformed fibroblasts produce only about 25% of the collagen produced by the normal fibroblasts (Table 1). The SVWI-38 cells displayed an overall decrease in total radioactivity deposited into the extracellular matrix, but the decrease in collagenase sensitive radioactivity was much more pronounced. Since [^{3}H]-proline was used to measure matrix components, the collagens would be labelled to a higher specific activity than would other proteins. After treatment of cells with aza C or aza CdR, no significant changes were observed in the amounts of collagen deposited into the extracellular matrix. Analysis of the collagens on polyacrylamide gels further

Table 1

Measurement of collagen deposition in the extracellular matrix (ECM) of WI-38 and SVWI-38 fibroblasts as well as SVWI-38 fibroblasts after treatment with aza C or aza CdR. Extracellular matrices were prepared as described in Methods.

Cell line	Total dpm in ECM	Collagenase sensitive dpm	% Collagen in ECM
WI-38	49242 ± 1222	16565 ± 238	34
SVWI-38	16854 ± 742	1474 ± 96	8.7
SVWI-38 azaC	18235 ± 618	1631 ± 121	8.9
SVWI-38 aza CdR	18413 ± 512	1473 ± 115	8.0

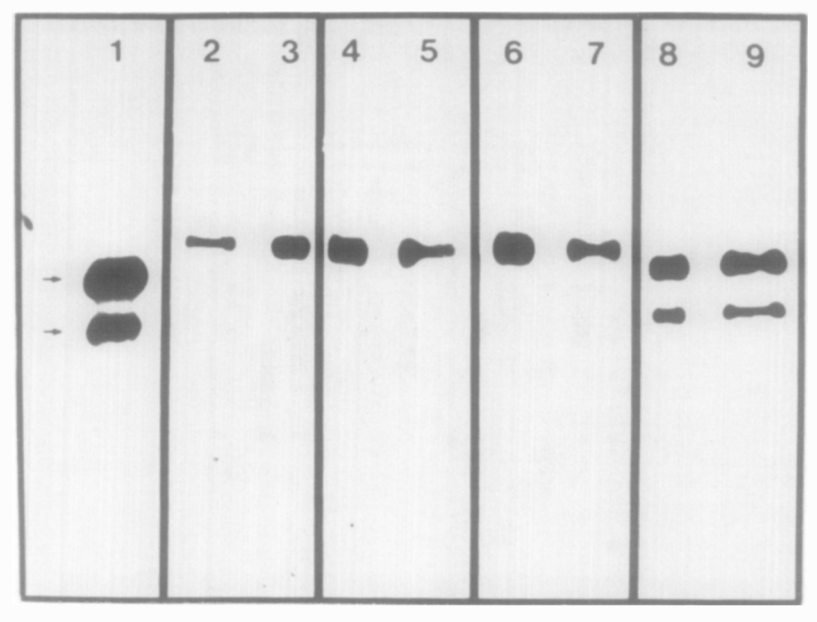

Figure 1. Collagen synthesis in WI-38 and SVWI-38 cells. Collagens were labelled and isolated as described in Methods. The arrows indicate the position of migration of the α1(I) and α2(I) collagen chains. Samples are collagens secreted by primary human embryonic lung fibroblasts (lane 1), SVWI-38 cells (lanes 2 and 3), SVWI-38 cells treated with aza C (lanes 4 and 5), SVWI-38 treated with aza CdR (lanes 6 and 7) and WI-38 cells (lanes 8 and 9). Odd numbered lanes are samples treated with β-mercaptoethanol.

indicated that no type I collagen was produced (Figure 1) under any of the conditions tested; thus treatment of the cells with aza C or aza CdR did not result in changes in the collagen types being synthesized. Also clear from Figure 1 is the fact that the major collagen produced by SVWI-38 was not disulphide bridged, thus ruling out the possibility that a significant amount was type III collagen. We have previously shown that no type I mRNAs are produced by SVWI-38 fibroblasts (11). Analysis of the α1(I) and the α2(I) genes revealed that these genes were hypermethylated in the SVWI-38 cells when compared with their normal counterparts (Figure 2). In the transformed cells the restriction endonuclease fragments between 0.5 and 2 kb were completely absent after digestion with HpaII or MspI, indicating methylation

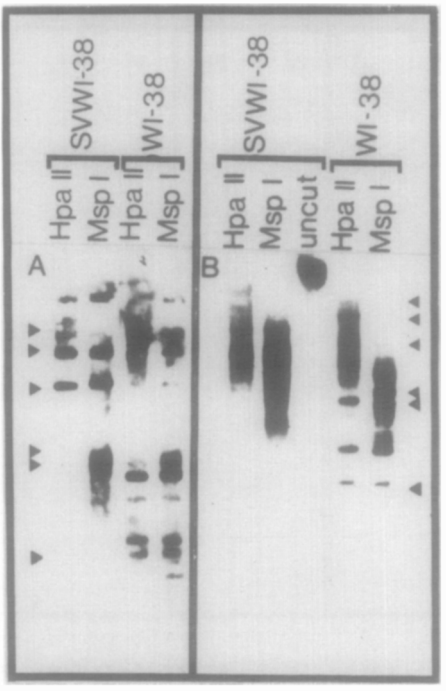

Figure 2. Methylation status of the α1(I) and α2(I) genes in normal and transformed cells. Samples were digested with the indicated restriction endonucleases and hybridized to either the α1(I) probe (A) or the α2(I) probe (B). Arrows indicate the position of migration of λ Hind III fragments.

of both cytosines in the CCGG recognition sequences, since neither of these isoschizomers will cleave if both cytosines are methylated. These results also showed that some cytosines were methylated even when the genes were active (i.e. in WI-38 cells).

Treatment of the cells with aza C or aza CdR resulted in gross demethylation of the total DNA as judged from ethidium bromide-stained gels (data not shown). Therefore, even though the clones used in this study covered the 3'-end of the type I procollagen genes, the observed demethylation would have been representative of what was occurring over the entire gene. Lower concentrations of the deoxy analogue were used since it would be incorporated more efficiently into the DNA than aza C and also, higher doses were found to be toxic to the cells. Analysis of

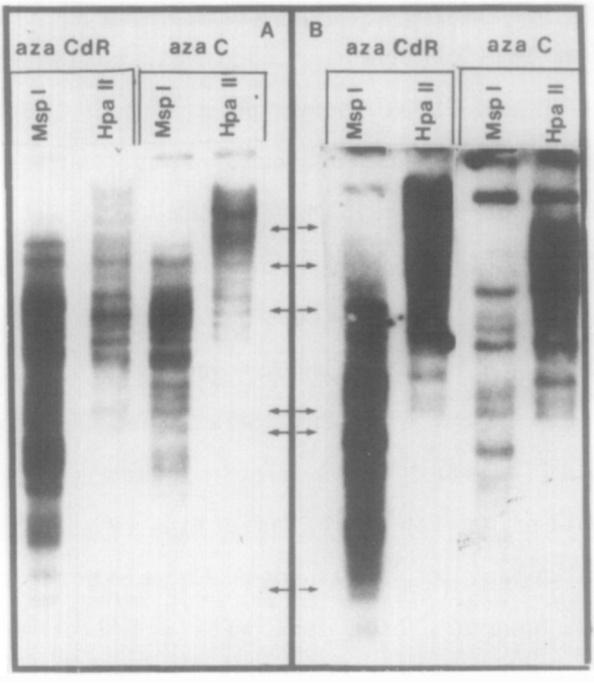

Figure 3. SVWI-38 cells were treated with either 5-aza C (30 μM) or 5-aza CdR (3 μM), the DNA isolated, and restricted with the indicated endonucleases. Samples were hybridized to either the α1(I) probe (A) or the α2(I) probe (B). Arrows indicate the position of migration of λ Hind III fragments.

the type I procollagen genes in SVWI-38 cells after analogue treatment revealed several bands in addition to those observed in normal cells (Figures 2 and 3). This finding confirms the fact that both cytosines in some CCGG sequences were methylated in cells expressing type I procollagen genes, and presumably did not play a significant role in gene regulation. These methylated sites would not have been detected in the normal HpaII/MspI analysis since neither of these enzymes would have been able to cleave them. Nevertheless, although these genes were extensively demethylated, the cells failed to produce type I collagen.

DISCUSSION

Malignant transformation of eukaryotic cells is usually accompanied by several changes in phenotypic expression. One of

the major changes observed is a decreased expression of several extracellular matrix components such as collagen (11,15,16), fibronectin (17) and hyaluronic acid (18). Such decreased synthesis of connective tissue components is associated with diminished cell-cell and cell-substratum interaction. In our studies to determine how the procollagen genes are affected during transformation, we attempted to reverse this shut-off of type I procollagen gene expression by administration of the cytidine analogues aza C and aza CdR. Aza C has been used to activate the normally dormant fetal globin genes in anemic baboons (8), and in patients with β-thalassaemia (9), as well as the metallothionein genes in a rat cell line where they are not expressed (19). However, treatment with aza C or aza CdR may be tissue and species-specific so that not all cells will respond in an identical manner. Even though the DNA became extensively demethylated in these WI-38 and SVWI-38 cells after aza C or aza CdR treatment, they did not undergo the phenotypic conversion observed in the case of mouse C3H/10T$\frac{1}{2}$ fibroblasts and some other cells (3).

Our results show that some cytosines in active genes are methylated even in expressing tissues, implying that if methylation plays a role in the regulation of gene expression, then only certain sites are crucial. Also evident is the fact that some other mechanism must also operate in conjunction with methylation of cytosines, since no type I collagen was synthesized in cells whose type I procollagen genes were extensively demethylated after aza C or aza CdR treatment.

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