Effect of 5-Azacytidine on Gene Expression in Marrow Stromal Cells

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When exposed to 5-azacytidine, marrow stromal cells from active long-term marrow cultures and cell lines derived from simian virus 40-transformed stromal cells rapidly upregulated *c-abl* and interleukin-6 transcripts while downregulating the expression of collagen I, a major matrix protein. Similar effects occurred with interleukin-1 α and tumor necrosis factor α , although the time course was considerably prolonged.

5-Azacytidine is a cytidine analog capable of altering the expression of certain genes, some of which may control differentiative programs. A widely held theory postulates that 5-azacytidine replaces cytidine residues within the DNA strand, where it then blocks the transfer of methyl moieties by DNA methyltransferase, resulting in de facto demethylation of the cytidine residues. Within certain GC-rich promoters, such demethylation in turn results in the activation of affected genes, although this activation is not universal among demethylated genes (8, 10, 12, 14, 15). Human genes which possess GC-rich promoters include "housekeeping genes" needed for routine cellular functions (12, 13, 16, 28, 30, 31, 34). The methylation patterns of some genes are tissue specific. Hence, it is not surprising that methylation plays a role in cellular differentiation, as has been demonstrated in multipotential murine mesenchymal cells. In these cells, 5-azacytidine initiates a complex polygenic differentiative program leading to the formation of mature myoblasts, chondrocytes, and adipocytes (18, 23, 33).

Marrow stromal cells are a heterogeneous population of mesenchymal cells necessary for the self-renewal, proliferation, and differentiation of hematopoietic stem cells in long-term marrow cultures (6). Both normal and cloned simian virus 40 (SV40)-transformed cultures of human marrow stromal cells upregulated the expression of the *c-abl* proto-oncogene in response to interleukin-1 α (IL-1 α) and tumor necrosis factor α (TNF α). There was a simultaneous increase in the expression of IL-6 from nondetectable levels and a downregulation of the major matrix protein, collagen I (D. F. Andrews, J. J. Nemunaitis, and J. W. Singer, submitted for publication). In the present study, similar effects were produced by 5-azacytidine.

Long-term marrow cultures were grown from marrow buffy-coat cells, and SV40-transformed cell lines were developed from confluent adherent cell layers (21, 29). All experiments were performed either with fresh 4-week-old confluent adherent cells or with transformed cell lines between passages 10 and 15. The substances tested for effects on gene expression included IL-1 α (10 U/ml) (32) (kindly donated by Immunex Corp., Seattle, Wash.) and TNF α (1,000 U/ml) (kindly donated by Genentech, San Francisco, Calif.). 5-Azacytidine (Sigma Chemical Co., St. Louis, Mo.) was applied to established cell cultures at concentrations of 1 and 10 μ M. Cells were harvested for DNA and RNA

extractions at 12 h, 24 h, and 3 days. To block RNA transcription, actinomycin D (Sigma) (7.5 μ g/ml) was added to selected cultures 6 h before RNA was harvested.

DNA and RNA were extracted (1, 19), and concentrations were measured by determining the optical density at 260 nm. The quality and quantification of whole-cell RNA was confirmed by running equivalent amounts on denaturing 1% agarose-formaldehyde gels and staining with ethidium bromide. Certain RNA samples were poly(A)⁺ selected over an oligo(dT) cellulose column (2). Northern (RNA) blotting and hybridization were performed (19), and autoradiograms were read on a densitometric scanner with MacIntosh-compatible software (Hoefer Scientific Instruments, San Francisco, Calif.).

DNA probes included a 1.5-kilobase (kb) *Bgl*II fragment isolated from the *v-abl* insert of the pABI sub 9 plasmid (35), a 1.8-kb collagen I fragment isolated from Hf677 (3), a full-length granulocyte-macrophage colony-stimulating factor (GM-CSF) cDNA, a 250 base-pair granulocyte colony-stimulating factor (G-CSF)-specific fragment, and a full-length IL-6 cDNA. (All growth factor cDNAs were generously provided by Immunex Corporation.) A glyceraldehyde-3-phosphate (6a) and a chick beta actin probe (4) were used as controls. Probes were labeled by either nick translation or random priming by using ³²P-tagged nucleotides (7, 26).

Three *c-abl* transcripts of 5, 6, and 7 kb are found in mRNA extracted from marrow stromal cells. The alternative first exons which initiate the 6- and 7-kb transcripts are preceded by a GC-rich promoter-like region (28) and thus might be derepressed by exposure to 5-azacytidine. On Northern blot analysis, 10 μ M 5-azacytidine increased the expression of all three *c-abl* transcripts but had a predominant effect on the 6-kb transcript (Fig. 1 and Fig. 2, top). In untransformed marrow stromal cells, *c-abl* upregulation was seen after 12 to 24 h of exposure to 1 μ M 5-azacytidine and continued to increase at 3 days (Fig. 1). When the experiment was repeated with cloned SV40-transformed stromal cell lines, maximal *c-abl* expression occurred after 12 h and transcript levels remained elevated at 24 h but fell to control levels by 72 h (Fig. 2). 5-Azacytidine at 10 μ M induced greater *c-abl* expression than it did at 1 μ M (data not shown). These findings were confirmed on slot blots (see Fig. 5). The inductive effect of 5-azacytidine on *c-abl* expression was comparable to the upregulation previously demonstrated with IL-1 α (10 U/ml), plus TNF α (1,000 U/ml) or with

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FIG. 1. *c-abl* expression in untransformed marrow stromal cells following 5-azacytidine induction. Whole-cell RNA extraction and hybridization procedures were performed as described in the legend to Fig. 2. Each lane contains 20 µg of whole-cell RNA.

phytohemagglutinin-stimulated T-cell-conditioned media (see Fig. 5) (Andrews et al., submitted).

The effect of 5-azacytidine on genomic *c-abl* DNA was studied through restriction analysis with the methylation-sensitive restriction endonuclease *Hpa*II and its isoschizomer, *Msp*I (Fig. 3). A Southern blot from marrow stromal cell DNA which had not been exposed to 5-azacytidine revealed that *c-abl* DNA showed different restriction patterns for the two enzymes, with few *Hpa*II restriction sites. After exposure to 5-azacytidine, however, *Hpa*II produced additional high-molecular-weight fragments, as well as low-molecular-weight fragments of 1.5 kb. The lower-molecular-weight restriction fragments were identical to those produced with *Msp*I, indicating demethylation within these regions. The relatively large amount of unrestricted high-molecular-weight DNA which persisted even after 5-azacytidine induction suggests that *c-abl* was demethylated in a relatively small percentage of cells, which were then responsible for the notable degree of *c-abl* transcript upregulation.

FIG. 2. Northern blot showing *c-abl* and collagen transcript expression in transformed marrow stromal cells following 5-azacytidine induction. Whole-cell RNA was extracted from SV40-transformed marrow stromal cells at the times indicated. Equivalent loading of lanes was verified by staining the gel with ethidium bromide prior to transfer to nitrocellulose. Each lane contains 20 µg of whole-cell RNA. The same blot was hybridized with a 1.5-kb *Bgl*II fragment isolated from the *v-abl* insert of the pABI sub 9 plasmid (top panel) (35) and rehybridized with a 1.8-kb collagen fragment isolated from Hf677 (5) after the previous probe was removed.

FIG. 3. Methylation patterns of the *c-abl* gene in marrow stromal cells after 5-azacytidine induction. DNA was extracted as previously described (19). A total of 10 µg of DNA was digested with either *Hpa*II or with *Msp*I by using 5 times the amount recommended by the manufacturer. The resulting digested DNA was run on a 0.8% agarose gel and transferred to nitrocellulose as previously described (19). The resulting Southern blot was hybridized under stringent conditions with the *v-abl* fragment described in the legend to Fig. 2.

There was no change in the *Hpa*II-*Msp*I restriction patterns from transformed stromal cells treated with IL-1 plus TNF, suggesting that they increase *c-abl* expression by a different mechanism (data not shown).

IL-1-treated SV40-transformed marrow stromal cells up-regulated GM-CSF and G-CSF expression over 2 to 3 days (Andrews et al., submitted). No message for these factors was detected in poly(A)⁺ RNA from 5-azacytidine-treated stromal cells. On the other hand, the expression of IL-6, a growth factor minimally expressed by unstimulated stromal cells but upregulated in both native and transformed stromal cells after exposure to IL-1 or TNF, became detectable 12 h after 5-azacytidine was added (Fig. 4). Beta actin, another

FIG. 4. IL-6 transcript expression in transformed marrow stromal cells following induction with 5-azacytidine. Poly(A)⁺ RNA was selected over an oligo(dT) column as described in reference 2 in order to minimize cross-hybridization of the IL-6 probe with rRNA (2). A total of 5 µg of poly(A)⁺ RNA was loaded per lane. The blot was hybridized with a full-length IL-6 cDNA probe. The blot was similarly probed with full-length GM-CSF cDNA and with 250-base pair G-CSF-specific fragment. (All growth factor fragments were generously provided by Immunex Corp.).

FIG. 5. Slot blots demonstrating levels of *c-abl* and IL-6 RNA after 12 and 24 h of induction with 10 μ M 5-azacytidine (lanes 2 and 3) or after 3 days of induction with IL-1 plus TNF (lane 6). A total of 7.5 μ g of actinomycin D per ml was added to induced cell cultures for 6 h immediately prior to harvest (lanes 4 and 7). Each slot contains 2 μ g of whole-cell RNA. A glyceraldehyde-3-phosphate-dehydrogenase (G3PD) probe served as a control (6a). Bands on the slot blots were then compared by densitometric scanning. Transcript half-lives were determined by comparing relative densities of IL-6 RNA transcript bands with and without actinomycin D. Total area under the curve was integrated for each individual graph. Each peak was calculated as a percentage of this total area (% AUC).

gene with a GC-rich promoter, also demonstrated upregulation, albeit to a lesser degree than IL-6 or *c-abl*.

In a separate experiment, transcript half-life was determined by slot blot analysis. Actinomycin D was added after cells were treated with either 5-azacytidine or IL-1 plus TNF (Fig. 5). The slot blots were then hybridized with IL-6 and *abl* probes. IL-6 transcript half-life was considerably longer in 5-azacytidine-induced cells than in the cells induced with IL-1 plus TNF, with half-lives of approximately 20 and 3 h, respectively. *c-abl* transcripts similarly showed an increased half-life of approximately 11 h after 5-azacytidine treatment, compared with 2 h after treatment with IL-1 plus TNF.

When marrow stromal cells were cultured with IL-1 plus TNF, the expression of collagen I, a major extracellular matrix protein in long-term marrow cultures, was downregulated (21; Andrews et al., submitted). 5-Azacytidine (10 μ M) similarly downregulates collagen I expression after 3 days (Fig. 2, center). This delay may be due in part to the long half-life of collagen I transcripts. Similar downregulation of collagen I was found in untransformed stromal cells from long-term marrow cultures treated with 5-azacytidine (data not shown).

It is unlikely that 5-azacytidine directly effects pro-collagen I transcription in stromal cells, since collagen I transcription in chondrocytes does not relate to the methylation status of the promoter (8). In contrast, the correlation of the findings of demethylation of *c-abl* genomic DNA on Southern blot after restriction with methylation-sensitive enzymes and the upregulation of the *c-abl* oncogene on Northern blot analysis suggest that upregulation of *c-abl* gene expression may be induced directly by demethylation. It is therefore conceivable that a proto-oncogene like *c-abl* might have a role as such a regulatory gene in controlling the gene expression of matrix proteins and growth factors within hematopoietic supporting tissues.

When stromal cells were exposed to IL-1 plus TNF, *c-abl* expression progressively increased over 5 days. In contrast, the 5-azacytidine effect on *c-abl* was rapid, suggesting that 5-azacytidine may increase *c-abl* expression by exerting an effect predominantly on gene transcription, while the lymphokines may act by posttranscriptional mechanisms. TNF and IL-1 predominantly upregulate the 5-kb *c-abl* transcript concomitantly with upregulation of the expression of G-CSF, GM-CSF, TNF, IL-1, and IL-6. 5-Azacytidine predominantly upregulates the 6-kb transcript and only increases the expression of IL-6. These differences suggest different mechanisms of transcript upregulation by IL-1 plus TNF as opposed to 5-azacytidine. 5-Azacytidine may act directly as a transcriptional upregulator for IL-6 as well as *c-abl*. However, it is also possible in these cells that a single gene, possibly *c-abl*, is upregulated by 5-azacytidine and that the activated, demethylated gene in turn affects the regulation of the second gene. It is also possible that expression of both genes is controlled by a third demethylated gene which affects either gene transcription or transcript half-life (17, 36).

Although only two *c-abl* transcripts are found in most hematopoietic cells (6 and 7 kb), marrow stromal cells were found to express a 5-kb transcript also found in blast cells from patients with chronic myelogenous leukemia (1). When marrow stromal cells were exposed to IL-1 plus TNF, the predominant effect was on the 5-kb transcript, while with 5-azacytidine, the expression of the 6-kb transcript was increased. It is therefore possible that cells such as marrow stromal cells or spermatocytes whose DNA is hypomethylated express increased amounts of the lower-molecular-weight *c-abl* transcript (11). It is also possible that expression of the low-molecular-weight transcript is not restricted to certain tissues but that the half-life of the transcript is so short in tissues other than human marrow stromal cells that it is not detectable. Our estimation of *c-abl* transcript half-life by slot blot affords information only with regard to the predominant transcript. We are unable to make definitive statements about less prominent transcript half-lives, since the various transcripts cannot be differentiated on slot blot. 3'-Untranslated regions of RNA transcripts have been found to affect transcript stability and thereby regulate transcript half-life (25, 27). A lower-molecular-weight 4-kb *c-abl* transcript in murine testis has a truncated 3'-untranslated region compared with the larger transcripts (20, 22, 24). Although the human 5-kb *c-abl* transcript is possibly analogous to the mouse 4-kb transcript, only limited data describing the human *c-abl* 3'-untranslated region are published (28). Speculations as to the role of the human 5-kb transcript and the significance of possible alterations in its 3'-untranslated region will require further structural data. The selective effects of inflammatory lymphokines and 5-azacytidine on *c-abl* expression in marrow stromal cells and the association

with changes in expression of other genes suggest the possibility of different *c-abl* transcripts affecting discrete polygenic programs.

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