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Alterations in c-abl Gene Methylation in Cells Transformed by Phagocyte-generated Oxidants

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SUMMARY: DNA from 10T1/2 cells transformed by activated neutrophils was analyzed for restriction length polymorphisms (RFLPs) in cellular homologues of retroviral oncogenes, and consistent RFLPs were found in MspI sites of the c-abl gene of all PMN-transformed cell lines. MspI digests probed with c-myc, v-Ki-ras, v-Ha-ras or v-mos showed no RFLPs, and none were observed in EcoRI, PstI, HindIII, BamHI, SmaI, Sau3a, MboI, HhaI, or TaqI digests probed with v-abl. Analysis of HpaII digests supports the conclusion that c-abl RFLPs result from differential methylation of the CCGG HpaII/MspI recognition sequence. MspI RFLPs in the c-abl gene may provide markers for oxidant-related genetic injury.

Evidence indicates an important role for oxidant-related injury in carcinogenesis (1-4). The toxic effects of oxidants and oxygen radicals on cells during inflammation (5-9) and reperfusion after ischemia (10,11) have helped to establish the importance of oxidant-related injury in many disease states. To investigate the relation between inflammation and cancer, we previously demonstrated that activated human phagocytes produce mutations in bacteria and mammalian cells, and that exposure of normal cells to activated phagocytes can bring about malignant transformation (12-15). We have also

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shown that reactive oxygen metabolites generated enzymatically in the absence of cells can elicit the same events by themselves (14-15). Studies from independent research groups have confirmed these observations, further establishing their validity (16-19).

Although phagocyte-generated oxidants cause profound genetic changes, their mechanisms of action and the properties of the target(s) are unknown. Because of the pervasiveness of oxidant-related injury in living systems, the physical and chemical interactions between oxidants and DNA are being studied to distinguish carcinogenic events from those that do not cause transformation. To study these questions, we examined several cell lines transformed with phagocyte-generated oxidants for restriction fragment length polymorphisms in genomic sequences related to transforming retroviral oncogenes, and we report data consistent with differential methylation of the c-abl gene.

MATERIALS AND METHODS

Transformation by activated neutrophils. The cell line used as the basis for these experiments is the 10T1/2 (clone 8) line derived originally from C3H/HEJ mice by Reznikoff, et al. (20). DNA from normal C3H/HEJ mice purchased from the DNA Resource, Jackson Laboratories, Bar Harbor, ME and from 10T1/2 cells served as controls for transformed cells.

Transformation experiments were performed on four separate occasions, and transformants were either propagated entirely in tissue culture or propagated in tissue culture after establishment of tumors in nude mice (15). Regardless of the transformation protocol, DNA samples were prepared from cells within six passages of the initial plating after transformation to minimize potential problems associated with long term passage. DNA samples were prepared also from 10T1/2 cells exposed to activated phagocytes exactly as described above but not transformed to control for phagocyte exposure.

Southern blot analysis. Genomic DNA was isolated from individual cell lines by the method of Maniatis et al. (21). Twenty ug samples of DNA digested as recommended by the manufacturer with a minimum of 5 units of restriction endonuclease per ug DNA were deproteinized, concentrated, quantitated, and electrophoresed in 0.6-1.2% agarose gels for 16-20 h at 30 v in 89 mM Tris-borate, 89 mM Boric acid, and 2mM EDTA. DNA was transferred by capillarity to Gene ScreenPlus (DuPont New England Nuclear Products, Inc., Boston, MA) according to the manufacturer's recommendations. Blots were hybridized with probe labeled with [^{32}P]dCTP and [^{32}P]dGTP by nick translation (21) at 65°C in buffer containing 10% dextran sulfate, 1% sodium dodecyl sulfate, 1 M NaCl. Filters were washed free of unreacted probe by washing successively with 2x SSC (1x SSC is 150 mM NaCl, 15 mM Na-citrate) at room temperature, 2x SSC, 1% SDS at 65°C, and 0.1x SSC at 50°C. X-ray films were exposed to blots at -70°C between Cronex intensifying screens.

Oncogene probes were purchased from Oncor Inc., Gaitherburg, MD. The v-abl probe derives from a 2.4 kb HincII fragment of the gene in the region encoding the tyrosine kinase. As purchased, the probe has a 600 base deletion from the original HincII fragment (Oncor, Inc., personal communication).

RESULTS

As a first step in characterizing genomic events associated with malignant transformation caused by phagocyte-generated oxidants, several isolated transformed cell lines were screened for restriction length polymorphisms (RFLPs) in cellular homologues of retroviral oncogenes. DNA from cell lines digested with restriction endonucleases with hexameric

recognition sites were analyzed by Southern blotting with several probes to oncogene homologues.

No RFLPs were noted when DNA from oxidant-transformed, normal 10T1/2, and 3-MCA transformed cells was screened with N-myc, v-Ha-ras, v-mos, and v-abl probes after digestion with EcoRI, PstI, HindIII, BamHI, and SmaI (data not shown). In every instance, the restriction patterns of normal and transformed cells were indistinguishable and specific to the individual probes. In view of these results, we tested for RFLPs in the same DNAs after digestion with enzymes with tetrameric recognition sites to increase the probability of observing rearrangements or polymorphisms.

Of the oncogene probes with which we analyzed MspI-digested DNA, v-abl was unique in that it showed consistent RFLPs in every cell line transformed by activated phagocytes. Southern blots of MspI and Sau3A DNA digests were hybridized with v-mos, v-Ha-ras, and v-abl probes to assay for novel restriction fragments in mouse loci homologous to these genes. No RFLPs were detected in the Sau3A digests with any probes (data not shown). However, after digestion with MspI, all oxidant-transformed lines showed the same apparent loss of 7.7 and 1.4 kb fragments and appearance of new 4.8 and 1.2 kb fragments (Fig 1). Furthermore, DNA from one oxidant-transformed cell line (Fig 1, lane 9) contained a novel 3.0 kb fragment. These polymorphisms were unique to cells transformed by oxidants in that they were absent from the DNA of C3H/HEJ mouse spleen, normal 10T1/2 cells, chemically-transformed cell lines (Fig 1), and from phagocyte-exposed but non-transformed 10T1/2 cells

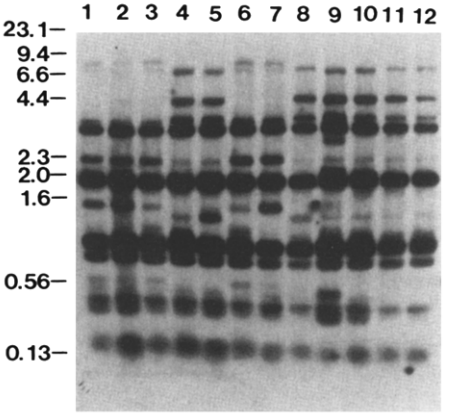

Figure 1. Restriction fragment length polymorphisms in MspI sites of the c-abl gene in oxidant-transformed cells. DNA samples digested with MspI were analyzed in Southern blots (Methods). Lanes contain DNA from the following: 1 and 3, untransformed control 10T1/2 cells; 2, C3H/HEJ mouse spleen; 4, 5, 8, 11, and 12, cells transformed with phagocyte-generated oxidants and established in mice (Methods); 9, and 10, cells transformed with phagocyte-generated oxidants (no passage through mice); 6 and 7, cells transformed chemically with 3-methylcholanthrene (no passage through mice). Lanes 11 and 12 contain DNA from the two related samples discussed in the text (Methods): 11=original benign tumor, 12=subsequent malignant tumor.

(not shown). The RFLPs were not an artifact of incomplete or inconsistent DNA digestion, because the patterns were reproduced in several experiments using different DNA preparations and different lots of restriction enzymes from different suppliers. Furthermore, no polymorphisms were observed in MspI digests probed with v-Ki-ras, c-myc, v-mos, or v-Ha-ras (data not shown), and the alterations were extremely consistent in that all oxidant transformed cells had some c-abl RFLPs, most of which were identical.

To test the specificity of these c-abl RFLPs for the MspI recognition site, Southern blots of TaqI, MboI, and HhaI DNA digests were also hybridized with v-abl probes. No RFLPs were observed in the TaqI or MboI digests (data not shown). In HhaI-digested DNA, the c-abl probe hybridized only to very high molecular weight and poorly resolved DNA fragments rather than to discrete bands (data not shown), but hybridization of c-myc to same blot showed that the DNA was digested completely (not shown), suggesting that GCGC HhaI recognition sites are extensively methylated in that portion of the c-abl gene assayed with the v-abl probe.

The apparent loss of both normal c-abl alleles from MspI digests of neutrophil-transformed DNA suggested that the RFLPs arose by methylation of sites within the c-abl gene, since consistent loss of more than one normal allele should be rare. To study the question of methylation further, DNA samples were digested with HpaII, an isoschizomer of MspI which is sensitive to methylation of the internal cytosine of the CCGG recognition sequence. In contrast to HpaII, MspI is sensitive to methylation of the 5′ cytosine but insensitive to methylation of the internal cytosine residue. As shown in Fig 2A, the v-abl hybridization patterns in HpaII digests were different for DNAs from each of the cell lines transformed by activated neutrophils. These data strongly support the conclusion that the c-abl gene of cells transformed by oxidants is variably methylated at the internal cytosine of CCGG sequences. Furthermore, since MspI is sensitive to methylation of the 5′ cytosine residue, MspI polymorphisms in the c-abl gene most likely result from consistent methylation of 5′ cytosines in the recognition sequence at the same position in c-abl.

DISCUSSION

On the basis of these results, we conclude that the c-abl gene contains specific CCGG sites that are particularly sensitive to methylation in association with oxidant-related transformation. Cells transformed by oxidants produced by activated phagocytes display very consistent changes in MspI sites of the c-abl gene. These polymorphisms appear to be restricted to CCGG sequences, because digestion with several restriction enzymes with other tetrameric recognition sites revealed no consistent polymorphisms.

As judged by the frequency of RFLPs in HpaII- and MspI-digested DNA, the polymorphisms seem to result from methylation of both cytosine residues in the

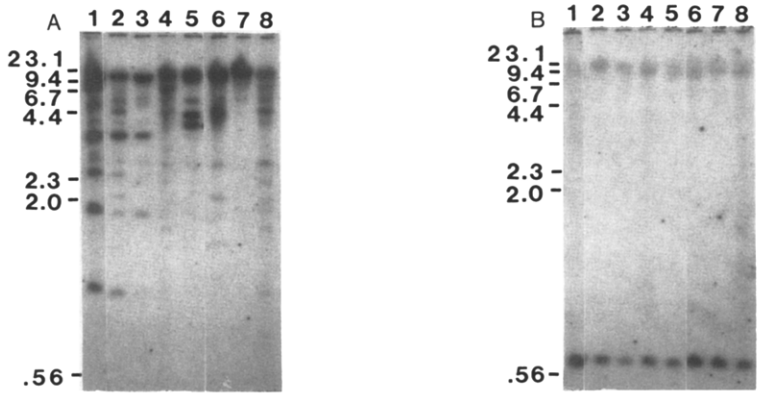

Figure 2. Southern blot analysis of HpaII-digested DNA hybridized with v-abl and with c-myc probes.

Panel A: v-abl probe. Lanes contain DNA from the following cells: 1, control 10T2/2, 2, transformed with 3-MCA; 3-8, transformed by phagocyte-generated oxidants.

Panel B: Same blot shown in panel A stripped and rehybridized with c-myc probe.

recognition sequence. These RFLPs are not seen in 10T1/2 cells exposed to phagocytes but not transformed (data not shown). Since HpaII and MspI polymorphisms occur independently of each other, the conclusion that individual oxidant-transformed cell lines contain c-abl genes that vary in the position and extent of cytosine methylation seems correct. The consistency of the MspI patterns shows that 5′ cytosine methylation of certain CCGG sites is almost invariant. On the other hand, the diversity of the HpaII patterns shows that methylation of the internal cytosine residue is highly variable among the different oxidant-transformed cell lines. Some specificity of these RFLPs for the c-abl locus is suggested by the absence of novel MspI fragments in DNA of the same oxidant transformed cell lines when probed with v-Ha-ras, v-Ki-ras, v-mos, and c-myc (data not shown). Thus, in some way, the RFLPs introduced into the v-abl homologue in these cells by their exposure to activated phagocytes occurs consistently, and, to the extent that these experiments have tested, specifically in CCGG sites.

c-abl is the cellular homologue of the transforming oncogene of the Abelson murine leukemia virus, v-abl (22-27). In the mouse, the highly conserved gene codes for a tyrosine-specific kinase that is essential for transforming activity (24). The physiological relevance of the oxidant-induced modifications observed in this gene is not known. Preliminary northern blot analyses show that phagocyte-transformed and control cells are not appreciably different with respect to overall levels of 6500 and 5300 nucleotide c-abl mRNAs (S. Weitzman, unpublished data), but studies using 5′-specific probes are needed to test for possible 5′ exon switching events (23). It seems quite probable that similar methylation changes might occur at other sites in the genome, perhaps in other protooncogenes.

The association of chronic inflammation with carcinogenesis has been recognized for centuries, but the first demonstration that activated phagocytes produced oxygen radicals occurred in the 1970's (6,28). The production of other oxidizing species, chloramines, for example, was described subsequently (29,30). The discovery that activated phagocytes produce the same products generated in water by ionizing radiation led to the hypothesis that phagocyte-generated oxidants play a role in development of cancer in association with inflammation. Phagocyte-generated oxidants cause breaks in DNA strands (31), are mutagenic in bacterial and mammalian cells (12,13,17-19), produce radiation-like modifications in nucleoside bases (32,33), convert endogenous lipids to genotoxic intermediates (34), introduce chromosome breaks (35), induce sister chromatid exchanges (14), act as promotors in certain initiation-promotion systems (2,4,16), can activate xenobiotic procarcinogens to ultimate carcinogens non-enzymatically (36), and can potentiate the carcinogenicity of xenobiotics in animals (37), and cause malignant transformation of mammalian cells (15). These observations support the idea that the mechanisms of transformation caused by oxidant-related injury are heterogenous and biologically relevant.

Oxidants generated by activated phagocytes produce phenotypic changes characteristic of carcinogens but the mechanisms through which oxidants induce cancer remain unknown. We now have shown that cells transformed *in vitro* by exposure to activated phagocytes or to oxygen radicals exhibit consistent covalent genomic modifications when compared to normal or chemically-transformed mouse cells. Our results show that altered methylation of c-*abl* CCGG sequences accompanies these transformation events, but the possible significance of these methylation changes to carcinogenesis remains to be elucidated. If other cells exposed to inflammation and subsequent transformation show the same *Msp*I-specific polymorphisms in the c-*abl* gene, these modifications may prove to be useful markers of oxidant-related transformation events, perhaps leading to an understanding of their role in carcinogenesis.

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