Epigenetic Silencing of the Human Nucleotide Excision Repair Gene, hHR23B, in Interleukin-6-responsive Multiple Myeloma KAS-6/1 Cells*

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During tumorigenesis, selective proliferative advantage in certain cell subsets is associated with accumulation of multiple genetic alterations. For instance, multiple myeloma is characterized by frequent karyotypic instability at the earliest stage, progressing to extreme genetic abnormalities as the disease progresses. These successive genetic alterations can be attributed, in part, to defects in DNA repair pathways, perhaps based on epigenetic gene silencing of proteins involved in DNA damage repair. Here we report epigenetic hypermethylation of the hHR23B gene, a key component of the nucleotide excision repair in response to DNA damage, in interleukin-6 (IL-6)-responsive myeloma KAS-6/1 cells. This hypermethylation was significantly abated by Zebularine, a potent demethylating agent, with a consequent increase in the hHR23B mRNA level. Subsequent removal of this drug and supplementation with IL-6 in the culture medium re-established DNA hypermethylation of the hHR23B gene and silencing of mRNA expression levels. The inclination of DNA to be remethylated, at least within the hHR23B gene promoter region, reflects an epigenetic driving force by the cytogenic/tumorigenic status of KAS-6/1 myeloma. The IL-6 response of KAS-6/1 myeloma also raises a question of whether thepreneoplastic growth factor, such as IL-6, supports the epigenetic silencing of important DNA repair genes via promoter hypermethylation during the development of multiple myeloma.

The process of DNA repair is designed to maintain genomic integrity, which is continuously challenged by damaging factors such as reactive oxygen species generated by normal cellular metabolism, errors in DNA replication, ultraviolet radiation, and chemical reagents (1). Failure in the repair of DNA damage introduces genomic mutations, and long term accumulation of these mutations leads to genomic abnormalities as seen in many human cancers.

In mammalian cells, nucleotide excision repair (NER) is one of the mechanisms for repairing DNA damage. The pivotal role of NER is to remove helix-distorting damage, including the UV-induced lesions (e.g. [6–4] photoproducts and cyclobutane pyrimidine dimers), interstrand and intrastrand cross-links, and chemically induced bulky DNA adducts. The NER process involves multistep mechanisms that require complexes of proteins to participate (1, 2). Xeroderma pigmentosum is one of the inheritable diseases associated with an NER defect which confers UV hypersensitivity and skin carcinogenesis, suggesting that a reduced gene expression of NER proteins would result in failure to maintain genomic integrity (1, 2).

Based on aberrant DNA hypermethylation, epigenetic silencing of genes involved in growth control is known to be associated with tumorigenesis. In most cases, DNA hypermethylation occurs in gene promoter regions, for instance, of the p53 gene in human breast ductal carcinoma (3) and human primary hepatocellular carcinoma (4) and of the O6-methylguanine-DNA methyltransferase gene in lymphomas (5). DNA methylation involves the transfer of a methyl group onto position 5 of the cytidine of CpG dinucleotides, whereas Cpa and Cpt dinucleotides are also observed (6).

Tissues undergoing infection, chronic irritation, and inflammation are rich in inflammatory cells, growth factors, activated stroma, and DNA damage-promoting reagents (7). Consequently, these microenvironments foster sustained cell proliferation and survival. This also suggests that certain pro-inflammatory cytokines required by the innate immune system are involved in the neoplastic process. IL-6 is one of the pleiotropic cytokines and regulates hematopoiesis, inflammation, and oncogenesis. We have demonstrated that IL-6-induced STAT3 activation initiates transcription of the transcription factor Fli-1 (Friend leukemia integration-site 1), and Fli-1 in turn activates the promoter and increases the expression of DNMT1 (DNA methyltransferase) (8, 9). In lymphocytes of patients with chronic lymphocytic leukemia as well as in acute myeloid leukemia, higher expression of DNMT1, -2, -3a, and -3b has been reported (10). However, epigenetic silencing via promoter hypermethylation has been seen only in a relatively small number of diverse genes that are important in DNA damage repair (5, 11). This might provide an explanation for the leukemogenesis arising from the clonal expansion of cells that have undergone successive alternations of genotypes (12, 13).

The abbreviations used are: NER, nucleotide excision repair; IL, interleukin; MM, multiple myeloma; ZEB, Zebularine-treated; NEG, untreated; REM, Zebularine-removed.

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considering that multiple myeloma (MM) is commonly associated with genomic alterations and that its development is IL-6-related (14), we hypothesized that the epigenetic silencing of DNA repair proteins, leading to chromosomal aberrations, may be one of the causes for the development of MM. Furthermore, this may be, in part, attributed to pro-inflammatory IL-6 signaling. We used the IL-6-responsive KAS-6/1 multiple myeloma to establish a link between IL-6-induced cell survival and DNA hypermethylation of DNA repair proteins, such as the hHR23B nucleotide excision repair protein, described in this study.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Drug Treatment**—The human KAS-6/1 cells were grown in RPMI 1640 media with 10% fetal bovine serum supplemented with IL-6 (10 ng/ml). Cells were treated with Zebularine (25 μM) for 48 h. After Zebularine treatment, the treated cells were washed three times with phosphate-buffered saline followed by culturing for 1 week in fresh medium with IL-6 supplementation (10 ng/ml). KAS-6/1 cells were kindly provided by Dr. Diane Jelinek (Department of Immunology, Mayo Clinic/Foundation, Rochester, MN). Zebularine was generously provided by Dr. Marquez (Laboratory of Medical Chemistry, National Cancer Institute, Frederick, MD).

**Analysis of Gene Expression by Ribonuclease Protection Assays and Real-time Quantitative Reverse Transcription-PCR**—Total RNAs from untreated cells, Zebularine-treated cells, and cells allowed to re-establish their methylation patterns following removal of Zebularine were extracted by using TRIzol reagent (Invitrogen). Ribonuclease protection assays were performed according to the manufacturer’s protocol. Briefly, α-32P-labeled multiple antisense RNA probes were synthesized in vitro by T7 RNA polymerase using the multiprobe DNA template. 20 μg of total RNA were hybridized to 2 × 106 cpm of labeled probes corresponding to the multiprobe template human NER-1 (human nucleotide excision repair set-1) (RiboQuant ribonuclease protection assay kit, BD Biosciences) overnight at 56 °C. Unhybridized RNA was digested first with RNase T1 for 30 min and then with RNase A for 45 min at 30 °C for 45 min at 37 °C. After phenol/chloroform extraction and sodium acetate/ethanol precipitation, hybridized RNA probes were denatured at 90 °C for 3 min and electrohoresed on a 6% polyacrylamide gel. The dried gel was exposed to an x-ray film.

The cDNAs were prepared by reverse transcribing 1 μg of total RNA using the single-strand cDNA synthesis kit (Roche Applied Science) according to the manufacturer’s protocol. Quantitative PCR analysis was performed using Taq Man probes (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions in 10-μl final volumes and in 384-well microtiter plates. Thermocycling conditions using an Applied Biosystems ABI-7900 SDS were as follows: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 10 s and 60 °C for 1 min. Specific primers for quantitative PCR were designed using the Applied Biosystems assay-by-design primer design software, and their sequences are proprietary in nature. The target mRNA expression was normalized to the 18 S expression, and the relative expression was calculated back to the untreated control.

**Chemical Modification of Genomic DNA**—EcoR I-digested genomic DNA (4 μg/μl) was denatured with 0.3 μM NaOH for 15 min at 37 °C and subsequently modified by 3.1 μM sodium bisulfite and 0.5 mM hydroquinone in 480 μl under mineral oil for 16 h at 55 °C. Modified DNA was purified using the QIAquick DNA extraction kit (Qiagen) and then treated with 0.3 μM NaOH for 15 min at 37 °C to complete the modification. After sodium acetate/ethanol precipitation, the modified DNA was resuspended in 50 μl of H2O for PCR amplification.

**PCR Amplification of Bisulfite-treated DNA**—Two sequential PCRs were used to amplify the modified DNA fragments of interest. 2 μl of modified DNA template was used in the first PCR (a). Nested primers were used in the subsequent PCR (b). The primers for the hHR23B gene (GenBank™ accession number AJ165178) are: (a) first PCR, 5'-GAA-TTATG(C/T)ATGATTTGTTGCGGTTGTT-3' (sense, nucleotides 721–750) and 5'-ATAAAAATAAAAAACCTTACATCTTTAAGCCCA-3' (antisense, nucleotides 1400–1430); (b) nested PCR, 5'-GGGTTTCTCT-GTATTTTTGTTGCGGTTGTT-3' (sense, nucleotides 841–870), and 5'-CACAACAAAATCTCCAAGCTTTACTTTGTTGTTGTTGTT-3' (antisense, nucleotides 1220–1252). PCR conditions for both PCR reactions were as follows: 95 °C for 30 s followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 2 min, and finally 10 min at 72 °C. The amplified DNA fragment was subjected to cloning using the pCR4-TOPO TA cloning vector (Invitrogen).

**RESULTS**

**hHR23B Gene Expression Is Elevated by Zebularine**—Considering karyotypic abnormality in MM, we postulated that nucleotide excision repair proteins are epigenetically silenced during the development of MM. To approach this, we used Zebularine, a demethylating agent that is reported to reverse the DNA methylation in the genome and, in doing so, to result in higher gene expression of hypermethylated genes. Survival of KAS-6/1 cells does not require IL-6; however, their growth is potentiated by the pro-inflammatory cytokine IL-6 (17). KAS-6/1 cells were evaluated under three experimental conditions: 1) untreated (NEG), 2) Zebularine-treated (ZEB), and 3) ZEB-treated cells rescued by IL-6 supplementation (REM) after removal of the drug. After removing Zebularine, the absence of IL-6 in the culture medium failed to sustain survival of the KAS-6/1 cells, leading to significant cell death.

Isolated total RNAs from NEG, ZEB, and REM KAS-6/1 cells were subjected to ribonuclease protection assay (Fig. 1). The expression profile indicated that among the nucleotide excision repair proteins, DDB1, hHR23B, and likely RPAp32 were up-regulated by Zebularine, genomic DNAs isolated from NEG, ZEB, and likely RPAp32 were up-regulated by Zebularine treatment (ZEB) compared with the untreated one (NEG). Intriguingly, these three up-regulated NER genes in ZEB were subsequently silenced when Zebularine was removed and cells were recultured in IL-6 (REM). Of the three candidate genes, the hHR23B gene, exhibited the most significant increase (approximately 5-fold).

**DNA Hypermethylation of the hHR23B Gene Is Associated with Transcriptional Silencing**—To confirm the demethylating effect of Zebularine, genomic DNAs isolated from NEG, ZEB, and REM KAS-6/1 cells were subjected to sodium bisulfite DNA sequencing. The investigated upstream region of the hHR23B gene corresponds to positions −360 to +55, relative to the transcription start site, and contains a high density of CpG dinucleotides (so-called “CpG islands”) (Fig. 2A).

The bisulfite DNA sequencing result of NEG shows the methylation status of 47 potential methylation loci (underlined) scattered from positions −338 to −64 upstream of the transcription start site (Fig. 2A). The methylation loci are mainly CpG dinucleotides, and to a lesser extent, Cpa dinucleotides. There is a lack of methylation loci from position −63 to +55 despite the presence.
of clusters of CpG and Cpa dinucleotides in this region. However, the methylation status in the region downstream of the transcription start site has been proposed to be independent of its methylation status in the region downstream of the transcription start site has been proposed to be independent of its transcriptional activity. The methylation status in the region downstream of the transcription start site has been proposed to be independent of its transcriptional activity.

**Hypermethylation of the hHR23B Gene Is Reversible**—The survival of KAS-6/1 cells requires the presence of IL-6 in the culture medium after Zebularine treatment is discontinued. The intriguing observation is that the hHR23B promoter region in REM is overwhelmingly remethylated at the cytosines of the CpG and Cpa dinucleotides. The overall methylation density accounts for 54%, in contrast to 22% of ZEB (Fig. 2B). The diversity of the methylated loci among randomly chosen clones in REM suggests that the rescued KAS-6/1 cells are equally derived from the diverse cell population treated with Zebularine. This rules out the possibility that subsets of cells unaffected by Zebularine have a better survival rate, dominating the rescued cell population. However, the re-established methylation loci are more distributed from positioned cytosines 10–22 and 40–47, and those of NEG are more homogeneous. The cause of this difference remains unclear.

The **Reversible Methylation Process Is Kinetically Associated with Transcriptional Down-regulation of the hHR23B Gene**—To further understand this different remethylated pattern, we monitored the remethylating (resilencing) kinetics of the hHR23B gene using quantitative PCR analysis (Fig. 3). As expected, the expression of the hHR23B gene was elevated in ZEB compared with NEG. However, withdrawal of Zebularine did not immediately repress the hHR23B gene expression. Increased expression of hHR23B was observed within 48 h (day 1 and day 2). This might be attributed to a recovery period from the potential cytotoxic effects of ZEB following drug removal. The sustaining expression level indicates that the hHR23B gene promoter is not yet remethylated, at least within 48 h. Nevertheless, this increased expression level was soon followed by a decreasing hHR23B gene expression. The required time frame of delayed gene repression was normally observed at day 3 after drug removal using the real-time quantitative reverse transcription-PCR technique.

The **reduction of hHR23B expression** results from remethylation of the hypomethylated hHR23B gene promoter (Figs. 1 and 2). The delayed repression of the hHR23B gene requires a longer time frame for active DNA methyltransferases to accumulate after Zebularine is removed. This could involve the attenuation of alleles containing the incorporated analog, Zebularine, while the whole cell population undergoes further cell divisions. The newly synthesized DNA methyltransferases are no longer covalently trapped by Zebularine and would remethylate those hemi-methylated alleles, of which the newly synthesized DNA strands are deprived of methyltransferase imprint. Similar kinetics for the gene expression of remethylation was previously demonstrated for the p16 gene by reverse transcription-PCR techniques in the human T24 bladder carcinoma treated either with Zebularine (19) or with 5-aza-2′-deoxycytidine (20). This also suggests that the remethylation is time-dependent, rather than cell cycle-dependent.

Comparison of the methylation density of the hHR23B gene promoter between NEG and REM (Fig. 2B) shows differences among those identified methylation loci. The regions of cytosines at positions 5–13 and 31–39 exhibit a lesser extent of hypermethylation in REM. Interestingly, the prediction of cis-acting regulatory DNA elements for transcription factor binding (bimas.dert.nih.gov/molbio/signal/) within the hHR23B promoter region indicates that the less hypermethylated regions in REM are highly G + C-rich, among which four high scores of transcription factor Sp1 binding sites (5′-GGGGGGAG-3′, 5′-GGAGGGCGAG-3′, 5′-GGAGGGCGAGC-3′, and 5′-GGGGGGGGAG-3′, boxed in Fig. 2A) are predicted. The consensus sequence suggested for Sp1 recognition is either 5′-GGGGGAG-3′ or 5′-GGGGGGGAGC-3′ and 5′-GGGGGACAG-3′. The fact that the highly G + C-rich regions are less liable to de novo meth-
ylation machinery, especially after being devoid of epigenetic marking by Zebularine, is interesting. The similar observation that the Sp1-like element protects a CpG island from de novo methylation in the adenine phosphoribosyltransferase gene in a mouse model has been reported (21, 22).

The Nuclear hHR23B Proteins and Their DNA Damage Repair Activity in KAS-6/1 Myeloma Cells—The first step of NER requires a complex containing both the hHR23B and xeroderma pigmentosum complementation group C (XPC) proteins to recognize DNA lesions of the genome. The nuclear localization of the hHR23B proteins has been shown in HeLa cells by van der Spek et al. (23). Our immunohistochemical investigation of the hHR23B protein in KAS-6/1 cells also demonstrates this protein to be predominantly in the nucleus, with lower intensity observable in the cytosol (result not shown).

The defects of NER activity in xeroderma pigmentosum patients contribute to the development of skin cancer as a result of failure to repair UV-induced DNA damage (15). To assign the NER function of hHR23B proteins in KAS-6/1 cells, we adopted the plasmid host cell reactivation assay frequently used for assessing DNA repair activity in xeroderma pigmentosum group studies (15, 16). We used the luciferase reporter gene plasmid (pRSVluc) that has undergone progressive irradiation at doses of 200, 400, and 600 J/m². By co-transfecting the irradiated plasmid pRSVluc with or without the pIRES2-hHR23B expression construct into KAS-6/1 cells, the reactivation of luciferase reporter gene activity indicates the NER function of hHR23B proteins. As shown in Fig. 4A, the damage of UV irradiation resulted in a decrease in luciferase activity proportional to increasing doses of UV irradiation in hHR23B Gene Hypermethylation in Myeloma Cells

FIG. 2. The hypermethylation status in the promoter region of the hHR23B gene. A, DNA sequence of the hHR23B gene (GenBank™ accession number AY165178) between positions −360 to +55, relative to the transcription start site. Forty-seven identified methylated cytosines among CpG and CpA dinucleotides are underlined. Four boxed sequences (5’-GGGCGGAG-3’, 5’-AGGCGGAG-3’, 5’-CCCCGCC-3’ (reverse direction), and 5’-GGGCGGAG-3’) represent binding sites for transcription factor Sp1 (see “Results”). B, the overall DNA methylation density of NEG, ZEB, and REM. Serial circles of individual clones display the methylation status (solid, methylated; open, unmethylated) of each cytosine nucleotide corresponding to the position as shown in A. In all NEG, ZEB, and REM samples, the heterogeneity of the methylated loci of the individual recombinant clones well represents the diversities. This reflects no bias to predominantly amplify a single copy of genomic DNA template during the PCR amplification.

The Nuclear hHR23B Proteins and Their DNA Damage Repair Activity in KAS-6/1 Myeloma Cells—The first step of NER requires a complex containing both the hHR23B and xeroderma pigmentosum complementation group C (XPC) proteins to recognize DNA lesions of the genome. The nuclear localization of the hHR23B proteins has been shown in HeLa cells by van der Spek et al. (23). Our immunohistochemical investigation of the hHR23B protein in KAS-6/1 cells also demonstrates this protein to be predominantly in the nucleus, with lower intensity observable in the cytosol (result not shown).
KAS-6/1 cells, either with or without introduction of exogenous hHR23B. However, introduction of exogenous hHR23B elevated luciferase activity by approximately 2-fold. The increase was also observed when the pRSVluc in absence of UV irradiation (0 J/m²) was co-transfected with the hHR23B expression plasmid. This is likely because of pre-existing DNA nicks or breakages of the pRSVluc plasmid that are repaired by the exogenous hHR23B proteins. At the median dose of 400 J/m² UV irradiation (Fig. 4B), the exogenous hHR23B proteins exhibited an increasing DNA repair activity (from 0.3 to 0.5 μg of the pIRES2-hHR23B construct). The DNA repair activity was elevated to 4.4, 27.3, and 92.0% by co-transfecting with 0.3, 0.4, and 0.5 μg of the pIRES2-hHR23B construct, respectively. Taken together, these results demonstrate that in KAS-6/1 myeloma cells the hHR23B proteins are functional in repairing the genome damage caused by irradiation and presumably by other damaging factors.

**DISCUSSION**

The region (position -338 to -64) of the identified methylation loci of the hHR23B gene overlaps the 200-bp region that usually embraces the basic promoter, where complexes of universal transcription factors and RNA polymerases bind. The overall demethylation by Zebularine treatment renders these loci unmethylated, inclusive of several potential Sp1 binding sites (5'-GGGGCGGAG-3', 5'-GAGGCGGAGC-3', 5'-CCCGC-CCC-3' (reverse strand), and 5'-GGGGCGGAG-3', boxed in Fig. 2A), and is accompanied by higher hHR23B gene expression. Therefore, the methylation pattern found in the NEG and REM samples is likely to account for the transcriptional repression of the hHR23B gene. The reversible methylation after removal of Zebularine also reflects the hypermethylation tendency of the hHR23B gene due to the tumorigenic status of the KAS-6/1 myeloma cells. The slight difference of distribution of methylation density, such as Sp1-like elements, in the hHR23B gene promoter in REM suggests a delayed hypermethylation in this region (21, 22). However, it is not clear whether the re-established methylation pattern via Zebularine-generated “de novo” behavior could be due to the epigenetically established methylation marking. This might involve different structural organization in the chromatin, abnormal chromosomal translocations, and dysregulated gene profiles of transcriptional factors/co-factors, as KAS-6/1 myeloma cells are further tumorigenetically developed from early B cell maturation. There is also a lack of reports addressing specific DNA de novo methylation patterns, probably because of the high redundancy of CpG islands at the genomic DNA level.

Chromosome translocation and gene dysregulation are two major cytogenetic abnormalities resulting from the multistep development of myeloma. Most chromosome translocation occurs in the IgH locus (14q32), with an incidence of 60–65% in intramedullary myeloma, 70–80% in extramedullary myeloma, and >90% in myeloma cell lines (24). The primary translocation appears because of errors accompanied by VDJ recombination, IgH switch recombination, or somatic hypermutation during normal plasma cell development. The unrepaired errors during chromosomal rearrangement contribute to genetic instability, and the chance for genetic instability is increased if
the DNA repair genes are silenced during MM development. To date only aberrant promoter hypermethylation of one DNA repair protein, O6-methylguanine-DNA methyltransferase, has been reported in lymphoma (5). The hypermethylation of the hHR23B gene is the first example of epigenetic change of NER in malignant MM. In contrast, our preliminary result also shows that the hypermethylation of the hHR23B gene promoter region of normal resting human B cells does not exist (unpublished results). Considering that multiple myeloma arises from a normal germinal center, it is possible that the epigenetic hypermethylation of the hHR23B gene contributes the genetic instability of MM development during early B cell maturation.

Dysregulations of growth control-related genes (c-myc, cyclin D1, p53, pRb, FAS, FGFR3 (fibroblast growth factor receptor 3), and bcl-2) are also known to occur in the development of myeloma malignancy (12, 13). The hHR23B gene is dysregulated as reported in this study. Moreover, the hHR23B protein has been identified as one of the apoptosis-associated proteins in human Burkitt lymphoma (25). It is also proposed that NER is involved in p53-dependent apoptosis, which requires the involvement of four proteins, xeroderma pigmentosum complementation groups D and B (XPD and XPB), p53, and p33PGK (isoform of the tumor suppressor gene ING1) (26, 27). Therefore, epigenetic silencing of the hHR23B gene would favor the clonal expansion of myeloma cells.

Among a complex of lymphohematopoietic growth factors involved in myeloma development, IL-6 supports the survival and/or expansion of MM cells not only by stimulating cell division but also by preventing apoptosis (28). In multiple myeloma, IL-6 survival and proliferation mechanisms are due to the activation of the Janus kinase/signal transducers and activators of transcription (JAK/STAT), phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt), and mitogen-activated protein kinase (MAPK) pathways (29–31). Little is known about whether the pro-inflammatory cytokine IL-6 contributes to epigenetic cellular gene silencing. Previous studies from our laboratory have demonstrated that IL-6 up-regulates DNMT1 (DNA methyltransferase), possibly through the transcription factor, Fli-1 (Friend leukemia integration-site 1), in human erythroleukemia K562 cells (8, 9). Using the IL-6-dependent myeloma KAS-6/1, we here demonstrated that the NER hHR23B gene is hypermethylated in the promoter region, as well as other tumor suppressor genes.2 The reversible DNA hypermethylation process in the presence of IL-6 releases the question of whether the prolonged proenoplastic effect of IL-6 is one of many factors contributing to the development from plasma cells to myeloma. Nevertheless, how this signaling directs hypermethylation, possibly through different transcription factors such as pRB and E2F1 (32), remains to be addressed.

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