Mechanism for Inactivation of the KIP Family Cyclin-dependent Kinase Inhibitor Genes in Gastric Cancer Cells

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Abstract

The mechanism for inactivation of the KIP family cyclin-dependent kinase inhibitor (CDKI) genes, the p21, p27, and p57 genes, in gastric cancer cells was tested by treating the cells with either the DNA demethylation agent, 5-aza-2′-deoxycytidine or the histone deacetylase inhibitor, n-butyric acid or trichostatin A. RNA expression of the gene was determined by reverse transcription PCR. The p21 gene was activated only by histone deacetylase inhibitor. The p57 gene was activated by histone deacetylase inhibitors in all of the gastric cancer cell lines and by 5-aza-2′-deoxycytidine in five of eight gastric cell lines. However, the p27 gene was not inactivated in gastric cancer cell lines. The methylation status of the promoter of the p21 and p57 genes was also tested by digestion with the methylation-sensitive restriction enzymes and a subsequent PCR. The promoter of the p21 gene has no methylation. The promoter of the p57 gene is, however, methylated in five of eight gastric cancer cell lines as expected from the result of the treatment with 5-aza-2′-deoxycytidine. Formation of the inactive chromatin through histone deacetylation seems to be the general mechanism for inactivation of both the p21 and the p57 genes in gastric cancer cells. Hypermethylation of promoter region seems to be an alternative pathway for inactivation of the p57 gene.

Introduction

Cell cycle progression is regulated by interactions between cyclins and CDKs (1, 2). Especially, the transition of G1 to S phase is known to be regulated by a family of negative cell cycle regulators, CDKIs. CDKIs are classified in two families, the CIP/KIP family and the INK4 family, based on primary sequence comparisons (3–5). The CIP/KIP family CDKIs, p21CIP1/WAF1, p27KIP1, and p57KIP2 share common sequence motifs that mediate interaction between CDKs and cyclin-CDK complexes (6–8). p21CIP1/WAF1 was the first CDKI to be identified, and it is known to be induced by p53, transforming growth factor β, differentiation, and cellular senescence (9). p27KIP1 is known to be involved in G1 arrest induction by cell-to-cell contact, cyclin AMP-inducing agents, and rapamycin (8). In contrast to ubiquitous expression of p21CIP1/WAF1 and p27KIP1, p57KIP2 is expressed at high levels in specific embryonic and adult tissue (7). Three genes, p21, p27, and p57, have been investigated in different kinds of human tumors and, unlike the INK4 family, only a few genetic alterations have been found. This suggests that the mutational inactivation of these CDKIs is infrequent (10), but gene inactivation by alternative mechanisms seems to be the general pathway.

Two known mechanisms, gene inactivation by methylation in promoter region and changes to an inactive chromatin by histone deacetylation, seem to be the best candidate mechanisms for inactivation of CIP/KIP family CDKI genes because these two mechanisms are frequently reported as the mechanism for the inactivation of specific genes. Histone acetylation results in the separation of DNA from histones, allowing euchromosomal DNA to become more accessible to transcription factors. The resulting histone hyperacetylation is correlated to the transcriptionally active state. Histone deacetylation allows the formation of normal nucleosome structure. This is referred to as transcriptionally inactive. The level of histone acetylation depends on the activity of histone acetyl transferases and HDACs. A number of genes and proteins have been identified for having activity as histone acetyl transferase or of HDAC. Naturally occurring compounds, such as n-butyric acid and TSA, are reported to inhibit the HDAC. They seem to induce general histone acetylation through a noncompetitive and nonspecific inhibition of the HDAC (11, 12). These compounds are known to induce the growth arrest and the differentiation in a variety of cancer cell types by activating the transcription of the p21 gene through Sp1 binding sites in its promoter (11).

Transcriptional repression by DNA methylation of promoters and 5′ regulatory sequences is expected to be the other pathway to inactivate the CIP/KIP CDKI genes. Changes in DNA methylation patterns are known to occur during tumorigenesis (13). CpG islands near promoters and 5′ regulatory region are usually unmethylated in normal somatic cells. In contrast, widespread methylation of CpG islands occurs on autosomal genes and leads to the silencing of the genes during oncogenic transformation (14). Promoters silenced by methylation can be reactivated by treatment with 5-aza-2′-deoxycytidine, which is a well-established inhibitor of DNA methylation (15). We hypothesized that abnormal DNA methylation and histone deacetylation might be the mechanism of inactivation of the CIP/KIP genes in gastric cancer cell lines. In this study, we report that histone deacetylation is a general mechanism for inactivation of the p21CIP1/WAF1 and p57KIP2 genes and methylation in promoter region is an alternative mechanism for inactivation of the p57KIP2 genes in gastric cancer cell lines.

Materials and Methods

Cell Lines and Cell Culture. Eight gastric cancer cell lines (SNU-1, SNU-5, SNU-16, SNU-620, SNU-638, SNU-668, SNU-719, and KATO-III) that were established from gastric carcinomas (16–18) were obtained from Korea Cell Lines Bank. Gastric cancer cells were cultured in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% (vol/vol) fetal bovine serum (Life Technologies, Inc.) and maintained at 37°C and 5% CO2.

Treatment of the Cells with HDAC Inhibitors or Demethylation Agents. To test the inactivation of the CIP/KIP family CDKI genes, gastric cancer cells (2 × 106 cells) in 100-mm-diameter dishes were treated with either the HDAC...
RESULTS AND DISCUSSION

To elucidate the mechanism of inactivation of p21, a KIP family CKD7 gene, in gastric cancer cells, eight gastric cancer cell lines were treated with either the HDAC inhibitor, n-butyl acid, or the demethylation agent, 5-aza-2′-deoxycytidine. RNA was isolated from cells and RNA expression was analyzed by RT-PCR. RNA expression of the p21 gene was activated only by the HDAC inhibitor, n-butyl acid, and not by 5-azac-2′-deoxycytidine in eight gastric cancer cell lines (Fig. 1A). The result suggests that the p21 gene is inactivated by formation of an inactive chromatin complex through histone deacetylation in gastric cancer cells. Methylation in the promoter of the p21 gene does not seem to be involved in inactivation of p21 in gastric cancer cells. The presence of methylation in the promoter of the p21 gene was further tested by digestion with a methylation-sensitive restriction enzyme and a subsequent PCR reaction. The GC-rich region in the six consecutive Sp1 binding sites of the p21 promoter was digested either with methylation-sensitive restriction enzyme and a subsequent PCR reaction. Because the promoter of the p21 gene is not methylated in gastric cancer cells. This confirms that methylation is not the mechanism for inactivation of p21 in gastric cancer cells.

To test the mechanism for inactivation of the p57 gene in gastric cancer cells, eight gastric cancer cell lines were treated with either the HDAC inhibitors, n-butyl acid or TSA, or the demethylation agent, 5-aza-2′-deoxycytidine. RNA was isolated from the cells and RNA expression was analyzed by RT-PCR. RNA expression of the p57 gene was activated by HDAC inhibitors, n-butyl acid or TSA, in all of the eight gastric cancer cells. SNU1 was an exception that TSA activated the p57 gene, but n-butyl acid did not. An appropriate explanation for this is not currently available except that it may be a clonal variation. Treatment with 5-aza-2′-deoxycytidine also activated the p57 gene in five of eight gastric cancer cell lines (Fig. 2). The result suggests that the p57 gene seems to be inactivated by the formation of an inactive chromatin complex containing HDAC. Methylation of the promoter of the p57 gene is also involved in inactivation of the p57 gene. The presence of methylation in the promoter of the p57 gene was further tested by digestion with a methylation-sensitive restriction enzyme and a subsequent PCR reaction. Because the pro-
motiv of p57 is highly GC-rich and has many MspI sites, a rare cutting and partially methylation-sensitive restriction enzyme, SacII was selected. Two SacII sites are located at −240 bp and −45 bp from the transcription initiation site. A primer set amplifies DNA across two SacII sites of the p57 promoter (Fig. 3A). Only undigested DNA will give a PCR product of 655 bp. DNA was isolated from untreated and 5-aza-2′-deoxycytidine-treated gastric cancer cells and digested with SacII. The resulting DNA was subjected to PCR (Fig. 3A). DNA from five untreated gastric cancer cell lines provided the PCR amplification products, but DNA from three untreated gastric cancer cell lines did not (Fig. 3B). DNA from all of the 5-aza-2′-deoxycytidine-treated gastric cancer cells did not make the PCR product. This indicates that five gastric cancer cell lines have the methylation in the promoter of the p57 gene. Treatment with 5-aza-2′-deoxycytidine removed the methylation from the promoter so that SacII cleaved the promoter, and PCR could not make the amplification product. The five gastric cell lines that yielded the PCR product were SNU-5, SNU-16, SNU-668, SNU-719, and KATO-III. These cell lines were exactly the same cell lines whose silent p57 gene was activated by treating with 5-aza-2′-deoxycytidine as shown in Fig. 2. All of the eight gastric cancer cell lines are established from gastric carcinomas. Six of them are established from metastatic ascites, and two of them are from primary carcinoma. Four of these five gastric tumor cell lines that are found to be methylated in the p57 promoter were established originally from metastatic tumors. Two of three tumors that are found to have no methylation in the p57 promoter were from primary tumors (16–18). This suggests that alteration in DNA methylation is acquired before tumors become metastatic. DNA methylation is an earlier event than metastasis in tumorigenesis. This explanation is in accord with the hypothetical model on molecular alterations in tumorigenesis of colorectal tumor proposed by Vogelstein. The extent of differentiation of original tumors and the extent of attachment in the growth of tumor cell lines were not correlated with the methylation status of the p57 gene in gastric tumor cell lines. The result suggests that formation of an inactive chromatin with histone and HDAC is a general mechanism for the inactivation of the p57 gene in gastric cancer cell lines because all of the gastric cell lines were activated by treating with a HDAC inhibitor. Methylation of the p57 gene seems to be an alternative way to form an inactive chromatin in gastric cancer cells.

To test whether the p27 gene, another Kip family CDK inhibitor, can also be activated, gastric cancer cells were treated with the same reagent, either the HDAC inhibitors, n-butyric acid and TSA, or a demethylation agent, 5-aza-2′-deoxycytidine. RNA was isolated from the untreated (C) and the drug-treated cells, was amplified by RT-PCR, and separated in 2% agarose gel as described in “Materials and Methods.” The size of the RT-PCR product was 287 bp (arrow). M, a DNA molecular weight marker.
low or no expression of RNA from either gene. Because expression of either the p21 gene or the p57 gene can cause the growth arrest, cancer cells seem to have the alteration in both genes to have advantage in their growth. Mutational alteration is rare in both genes, therefore, another possible mechanism is nonmutational inactivation because many developmentally regulated genes are inactivated without having mutation in adult tissues. Two known mechanisms are methylation of the gene and histone deacetylation. Although recent reports suggest that these mechanisms are overlapped (in that the binding of MeCP2, a methyl cytosine-binding protein, to methyl cytosine recruits the HDAC to inactivate the genes), they are not the same. Inactivation by histone deacetylation seems to be broader than the methylation-mediated inactivation. The histone deacetylation-mediated inactivation can include the methylated histone-mediated inactivation. However, the histone deacetylation-mediated inactivation does not necessarily involve the methylation of DNA. Our result supports this. The p57 gene is activated in all of the eight gastric cancer cell lines by the HDAC inhibitor, but it is activated in five of eight cell lines by 5-aza-2'-deoxycytidine. In the inactivation of p21, the methylation of DNA was not even involved. Methylation of the promoter is probably an alternative system to recruit the histone deacetylation. MeCP2, a methyl cytosine-specific binding protein, first binds the methyl cytosine and recruits the proteins including the HDAC to make an inactive chromatin complex as recent reports have described (20–22). In unmethylated DNA, recruitment of the HDAC by some transcription repressors seems to be involved. Transcription repressors like Mad, RB, YY1, and nuclear receptors are reported to recruit the HDAC (23–25). What is the corresponding factor in the p21 gene and the p57 gene? Sp1 binding sites are the common elements that exist in the promoters of both genes. Unfortunately, no such information is available for Sp1 or Sp1-binding proteins. Further characterization of the promoter and of the promoter-binding proteins in these genes will provide better understanding of the details of molecular level mechanisms of gene inactivation.

References
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