Impairment of Stromelysin-1 Transcriptional Activity by Promoter Mutations in High Microsatellite Instability Colorectal Tumors

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Abstract
Colorectal tumorigenesis is characterized by the sequential inactivation of a series of tumor suppressor genes (microsatellite-stable tumors) and genetic or epigenetic alterations in mismatch repair genes in nonpolyposic hereditary tumours and 13% to 15% of sporadic colorectal cancer [high microsatellite instability (MSI-H) tumors]. We hypothesized a molecular mechanism for MSI-H colorectal tumors related to matrix metalloproteinase 3 (MMP-3) promoter mutations, down-regulation of MMP-3 expression, and impairment of MMP-9 activation. We have now analyzed the 2.2-kb full MMP-3 promoter to assess the mutation distribution. The mutations found are restricted to the polymorphic region that includes the zinc-binding protein (ZBP-89) binding element. To show that these alterations were the cause of the low expression of this gene, we have generated three constructs with different MMP-3 promoters (wild type and two mutants) and we have expressed them in SW480 human colorectal cells. The basal transcriptional activity of wild-type MMP-3 promoter was much higher than the mutants activity. In addition, 12-O-tetradecanoylphorbol-13-acetate (TPA)–induced transcriptional activity of wild-type MMP-3 promoter was 10-fold higher than the mutants activity. Dexamethasone inhibited the basal transcriptional activity of wild-type MMP-3 promoter and of the two mutants found in the MSI-H subgroup of colorectal tumors. Significantly, dexamethasone almost completely blunted the TPA-induced effect on wild-type MMP-3 promoter transcriptional activity and on the mutants, even below their basal activity. Our data show that mutations found in the polymorphic region of the MMP-3 promoter from MSI-H colorectal tumors impair its basal and induced transcriptional activity, which may contribute to their better clinical outcome. (Cancer Res 2005; 65(9): 3811-4)

Introduction
Colorectal cancer represents a major public health problem in western populations. The colorectal tumorigenesis process is characterized by multiple mutations in common oncogenes and tumor suppressor genes, as well as genomic instability attributable to mismatch repair gene defects (1). Two main genetic pathways leading to colorectal cancer can be distinguished. The first and more common pathway is characterized by the sequential inactivation of a series of tumor suppressor genes, and the second genetic pathway is involved in the development of tumors from patients with hereditary nonpolyposis colorectal cancer (HNPCC) and in ~ 13% to 15% of sporadic large bowel adenocarcinomas (2-4). The hallmark of this alternative “mutator” pathway is widespread microsatellite instability (MSI). MSI evolves through mutations or epigenetic alterations of the DNA mismatch repair genes (5). Carcinomas with high MSI (MSI-H) develop an increased mutation rate and are believed to display distinctive pathologic features and to behave less aggressively than stable tumors (MSS; ref. 6). Thus, similarly to HNPPC, sporadic MSI-H tumors seem to have a better prognosis. The majority of these tumors are localized to the right colon (7, 8) and have better survival after adjuvant chemotherapy in colorectal cancer (9).

Stromelysin-1 [matrix metalloproteinase 3 (MMP-3)] is a member of the family of metalloproteinases, a group of enzymes that are responsible for the degradation of extracellular matrix components, such as collagen, laminin, and proteoglycans. These proteases are involved in normal physiologic processes, such as embryogenesis and tissue remodeling, and also play a significant role in cancer, arthritis, periodontitis, and other diseases (10-13). MMP-3 is secreted as a zymogen (59 or 57 kDa), which proteolytically processes to the 45- and 28-kDa active forms. This enzyme degrades a wide spectrum of extracellular matrix proteins, including type IV and IX collagen proteoglycans, fibronectin, and laminin. MMP-3 also activates proteolytically MMP-9 and other metalloproteinases and processes E-cadherin to an inactive form (14, 15). MMP-3 is thought to play an important role in pathophysiologic degradation processes associated with conditions, such as rheumatoid arthritis and cancer cell invasion (16).

In our previous study, we hypothesized a differential molecular mechanism between the MSI-H and MSS colorectal tumors, which may provide an explanation for their different clinical outcome. We found that the MSI-H group of tumors showed lower activation of MMP-9 compared with MSS tumors; this fact was related to much lower MMP-3 expression levels and to the presence of mutations in the MMP-3 promoter. These mutations were mainly deletions and/or insertions affecting repeated sequences located in this region. In fact, (T)5/(T)6 in 698 to 703 nucleotides and (C)6 between 704 and 709 nucleotides were the most affected sequences. All MSI-H tumors showed abnormalities in that region when comparing the sequences with the corresponding normal tissues (17).

In this study, our goal was to show that those mutations are the cause of the low expression of MMP-3. To achieve this goal, we have created different constructions with the wild-type and mutant MMP-3 promoters and transfected them into human cells to assess their transcriptional activity.

Materials and Methods
The starting point of this study was a previously characterized colorectal cancer population (17), obtained from the Hospital Clínico San Carlos, Madrid. This population was classified into two different subgroups (MSI-H...
containing 5% CO2 in air. The transfection was carried out when the cells from patient 12 corresponded to a showing different mutation levels in MMP-3(mut2) from patient 12. The reason for choosing two different promoters was to assess if there was a minor mutation level as compared with mut1. The reason that we had detected in MMP-3(mut1) from patient 2 displayed the higher mutation level than a construct containing 5T. Later, it was suggested that the 5T/6T polymorphic site was present in our MSI-H tumors, as well as in a pool of MSI-L/MSS tumors that were used as controls. The 5T/6T polymorphic site constitutes a repeated mononucleotide locus that could be a target for the mutator phenotype cancer pathway. Based on this, we investigated possible alterations of this region in the subgroup of sporadic colorectal MSI-H tumors, regardless of the subgroup of treatment, dexamethasone treatment, and dexamethasone and TPA combined treatment.

Under basal conditions, in the absence of TPA or dexamethasone, we found that the expression of the protein driven by the wild-type promoter was much higher than when it was driven by the mutated MMP-3 promoters. Data obtained from six independent experiments were as follows (arbitrary units, median ± SE, corrected with the internal control): 84.92 ± 2.57 for the pGL3-MMP-3(wt) construction, 23.94 ± 1.05 for pGL3-MMP-3(mut1), and 37.99 ± 0.94 for pGL3-MMP-3(mut2). Differences among groups were statistically significant (P < 0.001 for the three pairs wt-mut1, wt-mut2, and mut1-mut2).

The next condition that we studied was the expression of the MMP-3 promoters after TPA treatment. In every case, we detected an increase in the luciferase expression compared with basal levels. Data from six independent experiments were 1,465.87 ± 62.89 for pGL3-MMP-3(wt), 113.53 ± 6.74 for pGL3-MMP-3(mut1), and 112.95 ± 6.07 for pGL3-MMP-3(mut2). The response to the TPA induction was much higher in the construction driven by the wild-type promoter than in those driven by the mutated promoters. Differences among the expression of pGL3-MMP-3(wt) and mutated promoters were statistically significant (P < 0.001 in all cases wt-mut1 and wt-mut2).

The third condition analyzed was the luciferase expression upon treatment with dexamethasone. We observed a decrease in the level of expression compared with basal data in the case of the wild-type promoter and the mut2 promoter but not in the mut1 (highly mutated promoter) case. Data obtained from six independent experiments were 51.05 ± 2.48 for pGL3-MMP-3(wt), 42.38 ± 2.86 for pGL3-MMP-3(mut1), and 2.67 ± 0.42 for pGL3-MMP-3(mut2) (P < 0.05 for the wt-mut1 pair and P < 0.001 for the other two pairs).

Finally, we determined the luciferase expression upon TPA and dexamethasone treatment. Our data showed that the dexamethasone almost completely blunted the TPA-induced effect on the basal transcriptional activity driven by the wild-type promoter (157.51 versus 1,465.87, respectively; basal activity, 84.92). Dexamethasone completely prevented the TPA-induced effect on transcriptional activity driven by mutated promoters pGL3-MMP-3(mut1) and pGL3-MMP-3(mut2), even below their basal activity (9.43 versus 113.53 and 16.90 versus 112.95, respectively). All differences observed were statistically significant. Data on MMP-3 transcriptional activity driven by the wild-type, mut1, and mut2 promoters, described above, are shown in Figs. 2, 3, and 4, respectively.

**Discussion**

The presence of a common polymorphism in the MMP-3 promoter that was associated with progression of atherosclerosis was reported by Ye et al. (18). They showed that expression of the MMP-3 construct with 6T at the polymorphic site was much lower than a construct containing 5T. Later, it was suggested that the presence of the 5T/6T polymorphism at the MMP-3 promoter might be one of the risk factors for the development and/or progression of colorectal cancer (19, 20).

The region of MMP-3 promoter, located between nucleotides 699 and 704 (Genbank, GI: 11093513), constitutes a repeated mononucleotide locus that could be a target for the mutator phenotype cancer pathway. Based on this, we investigated possible alterations of this region in the subgroup of sporadic colorectal MSI-H tumors, as well as in a pool of MSI-L/MSS tumors that were used as controls. The 5T/6T polymorphic site was present in our colorectal tumor populations, regardless of the subgroup of...
tumors. However, we found $\text{MMP-3}$ promoter C, T, and G deletions and/or insertions in repeat sequences that were observed in all MSI-H tumors compared with their controls. None of the MSI-L/MSS tumors that were analyzed showed these alterations. These $\text{MMP-3}$ promoter mutations were correlated with a much lower expression of $\text{MMP-3}$ and a lack of activation of MMP-9 in MSI-H colorectal tumors compared with controls and with the MSS colorectal tumors. Finally, we proposed the $\text{MMP-3}$ promoter as a novel target of the defective mismatch repair machinery associated with MSI-H sporadic colorectal cancers (17).

We have now analyzed the 2.2-kb full $\text{MMP-3}$ promoter to assess the distribution of the mutations previously described in the MSI-H subgroup of tumors. These mutations are essentially constrained to the polymorphic region described above and also to all tumors grouped as MSI-H. This region is also termed SIRE. The SIRE site has been identified as a repressor of $\text{MMP-3}$ expression induced by IL-1. Mutations of two Cs eliminate binding and increase cytokine-induced transcription from the $\text{MMP-3}$ promoter (21). In addition, a 5T/6T polymorphism was described; the 5T promoter construct showed an increased transcriptional activity compared with a 6T construct. These data suggested that the repressor likely binds to the 6T site with higher affinity than to the 5T (18). This region has also been described to bind the transcriptional factor zinc-binding protein (ZBP-89), which up-regulates its activity. Its expression is increased in gastric carcinomas, induction of $\text{MMP-3}$ expression being a significant factor in tumor metastasis (22).

We have also studied the transcriptional activity of the wild-type $\text{MMP-3}$ promoter and two mutants (mut1 and mut2) found in the MSI-H subgroup of colorectal tumors. With that aim, we transfected the corresponding construct into human cell lines. First, we did the transfection into human HELA cells. However, the basal transcriptional activity found was very high, regardless of the construct. Then, we transfected SW480 human differentiated colorectal cells. The basal transcriptional activity of wild-type $\text{MMP-3}$ promoter was much higher than that of the mutants. These results are consistent with our current hypothesis that the $\text{MMP-3}$ promoter mutations in MSI-H sporadic colorectal tumors. Boxes show the alterations in the mut1 and mut2 promoters compared with the wild-type $\text{MMP-3}$ promoter and to a MSS tumor promoter. The shadowed sequence is the ZBP-89–binding element.
mutations found in the MSI-H subgroup of colorectal tumors may account for their decreased MMP-3 expression.

Downstream the ZBP-89 binding site, several regulatory elements have been described in the MMP-3 promoter. Among them, there are activator protein (AP-1) regulatory elements much closer to the transcription initiation site. Indeed, growth factors such as platelet-derived growth factor, through AP-1 transcription factor, have been involved in the up-regulation of MMP-1 and MMP-3 gene expression in fibroblasts. However, the full effect required the signaling from IL-1 through the transactivation effect of nuclear factor κB (NF-κB; ref. 23). Then, we studied the effect of phorbol esters (TPA) on the basal MMP-3 transcriptional activity. The TPA-induced transcriptional activity of wild-type MMP-3 promoter was 10-fold higher than that of the mutants. Our data strongly suggest that MMP-3 promoter mutations found in the MSI-H subgroup of colorectal tumors confer transcriptional inactivation, providing further support to their direct contribution to the decreased MMP-3 expression observed in those tumors.

Dexamethasone inhibited the basal transcriptional activity of wild-type MMP-3 promoter and also the two mutants (mut1 and mut2) found in the MSI-H subgroup of colorectal tumors. More importantly, dexamethasone almost completely blunted the TPA-induced effect on wild-type MMP-3 promoter transcriptional activity, even below the basal activity on the mutants. Recently, it has been described that the polymorphic region that binds to the transcriptional factor ZBP-89 also comprises a binding site to NF-κB in response to proinflammatory signals such as IL-1 (24). Thus, NF-κB and ZBP-89 may play an opposite effect on MMP-3 promoter, NF-κB limiting, as a repressor, the cytokine-induced MMP-3 expression. In the presence of proinflammatory stimuli, however, NF-κB transactivated MMP-3 expression as described in vascular smooth muscle cells in response to synergistic effect of growth factors and IL-1α and also in human synovial macrophage in response to tumor necrosis factor α (25, 26). Thus, dexamethasone through its anti-inflammatory pathway may down-regulate NF-κB expression, impairing the transcriptional activation of MMP-3 from either the wild-type or mutated promoters.

In conclusion, MMP-3 promoter mutations, constrained to its polymorphic region, from tumors grouped as MSI-H impair basal and induced transcriptional activity, which may contribute to the better clinical outcome of those tumors.

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