Sp1 Regulates Cathepsin B Transcription and Invasiveness in Murine B16 Melanoma Cells

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Abstract. Background: Increased expression of cathepsin B contributes to extracellular matrix degradation and invasion in cancer. Cathepsin B expression is under transcriptional control in murine melanomas and the major promoter contains potential binding sites for the Sp1 transcription factor. Materials and Methods: Murine melanoma cells transfected with an Sp1 expression plasmid or its control were used in Matrigel invasion and cell motility assays in the presence or absence of the cathepsin B inhibitor, CA-074Me. Results: Transfection of B16F1 cells with the Sp1 expression plasmid resulted in a 2.5- to 5.3-fold increase in cathepsin B specific activity and a 4.8- to 5.5-fold increase in invasiveness over the control, but had no effect on the movement of cells across an uncoated membrane. CA-074Me treatment resulted in significantly reduced Matrigel invasion without affecting cell motility. Conclusion: Sp1 can regulate the capacity of B16F1 cells to degrade a reconstituted extracellular matrix in part by regulating cathepsin B expression.

Cathepsin B is a lysosomal protease whose expression is frequently elevated in cancer (1). An imbalance between cathepsin B and its endogenous inhibitors contributes to the invasiveness of tumors and to a poorer clinical outcome for patients (1-5). Molecular genetic manipulations of cathepsin B expression have provided direct evidence that cathepsin B can contribute to the capacity of tumor cells to invade an extracellular matrix (6-8). The use of specific inhibitors of cathepsin B and confocal fluorescence microscopy to view invading cells suggest that cathepsin B degrades matrix components that are internalized by endocytosis, or in immediate contact with the surface of cells (8, 9). In murine B16 melanoma variants, cathepsin B expression and metastatic potential are highly correlated (2). The higher level of cathepsin B in the metastatic B16a melanoma is due to increased gene transcription, and a DNA fragment from the 5’ end of the mouse cathepsin B gene is capable of conferring the differential gene activation seen among B16 melanoma variants of differing metastatic abilities (10). This DNA segment is GC-rich and contains binding sites for the Sp1 and Sp3 transcription factors. In addition, our studies indicate that Sp1 is elevated in B16a melanoma cells compared to its poorly metastatic B16F1 parent (submitted for publication by Ahmed and Frankfater, 2004). Here, we show that introducing Sp1 into the poorly invasive B16F1 melanoma activates expression of the endogenous cathepsin B gene and produces an increase in the ability of these cells to invade a reconstituted basement membrane (Matrigel) layer.

Materials and Methods

Transfection of tumor cells. B16F1 cells were obtained from Isaiah J. Fidler (M. D. Anderson Cancer Ctr., Houston, TX, USA) and cultured as described previously (8). The plasmid pPacSp1, which contains a 2.1 kbp cDNA corresponding to a functional form of the human Sp1 transcription factor, and the empty vector pPac0, were the kind gifts of Dr. Robert Tjian (11). Cells were transfected using LIPOFECTAMINE (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions as previously described (8).

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Key Words: Cancer, invasion, Sp1 transcription factor, cathepsin B, Matrigel, CA-074.

Abbreviations: CA-074Me, (L-3-trans-propylcarbamoyloxirane-2-carboxyl)-L-isoleucyl-L-proline methyl ester; CBZ, benzoxycarbonyl; dNTP, deoxynucleotide triphosphate; DTT, DL-dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid; PET, polyethylene terephthalate; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

Electrophoresis and Western blot analysis. Nuclear extracts from B16F1 cells were prepared as described previously (12) and stored in 25-μl aliquots at -80°C. Proteins (7 μg/lane) were separated by electrophoresis on a 12% SDS-PAGE gel, transferred to nitrocellulose membrane (Nytran), and probed with an affinity purified, Sp1-specific, rabbit polyclonal IgG (sc-59, Santa Cruz Biotechnology, Santa Cruz, CA, USA 1:1000 dilution) and a peroxidase-conjugated goat anti-
contained 5 μl of a 10× PCR buffer, 1.5 mM MgCl₂, dNTPs (0.2 mM) PCR reactions were carried out in a final volume of 50 μl and was isolated and reverse transcribed as described previously (8). All measured by the reverse transcription-linked PCR. Total cellular RNA was harvested 36 h after transfection and 7 μg of a nuclear protein extract from each transfected was subjected to SDS polyacrylamide gel electrophoresis and immunoblotting. Sp1 was detected with a 1:1000 dilution of the Sp1-specific rabbit polyclonal IgG; α59, and a 1:2000 dilution of a peroxidase-conjugated goat anti-rabbit IgG, as described in Materials and Methods.

4-Methylcoumarin Hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) as previously described (2, 8). Cathepsin B mRNA was measured in cell lysates with the fluorogenic substrate N-α-CBZ-L-Arg-L-Arg 7-Amido-3888 (Kirkegaard & Perry, Gaithersburg, MD, USA). Light emission from treated membranes was detected with X-ray film (Sterling X-ray film, Dublin, OH, USA).

Measurements of cathepsin B. Active cathepsin B was measured in cell lysates with the fluorogenic substrate N-α-CBZ-L-Arg-L-Arg 7-Amido-4-Methylcoumarin Hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) as previously described (2, 8). Cathepsin B mRNA was measured by the reverse transcription-linked PCR. Total cellular RNA was isolated and reverse transcribed as described previously (8). All PCR reactions were carried out in a final volume of 50 μl and contained 5 μl of a 10× PCR buffer, 1.5 mM MgCl₂, dNTPs (0.2 mM each), primers (10 pmol each), 1 unit Taq DNA Polymerase (Invitrogen), and 1 μl of the reverse transcription reaction mixture. This mixture was incubated for 3 min at 94°C, and subjected to 25-40 cycles of amplification (94°C for 1 min, 56°C for 1 min, and 72°C for 1 min). The primers used to detect mRNAs from putative alternative transcription start sites were as follows: exon 1A, 5’AGTACGCGGAGAGGGCCA (14), exon 1B, 5’GCCATTCTTCCAAA GTTCTTGTTCGTC, or exon 1C, 5’CCGAGTCACTGCCGGGTGT GTAGAAA (15). The 3’ primer, 5’AGAGGATAGCCACCA GTACCAGCCGT, corresponded to a common sequence across the junction of exons 5 and 6 (14). Mouse actin mRNA was detected with the respective 5’ and 3’ primers, 5’GTGGGCCGCCCTAGGCACCA and 5’ CTCCTTGTAGTC AGCCAGATTTC (16). The PCR products were separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and the individual bands quantified by densitometry using photographic negatives. Measurements of cell invasion and motility. Cell invasion and motility were measured by in vitro assays as previously described (8). Invasiveness was determined by measuring the capacity of tumor cells to traverse a PET membrane filter (8 μm pores) coated with a reconstituted Matrigel basement membrane matrix (Becton Dickinson Labware, Bedford, MA, USA). Motility was measured in the same assay system using uncoated PET membranes. In some experiments, the intracellular cathepsin B specific inhibitor CA-074Me (10 μM, Peptide Institute, Inc., Osaka, Japan) was added to the invasion chamber at the beginning of the incubation period.

Results

Effect of Sp1 on cathepsin B gene transcription in B16F1 melanoma cells. B16F1 cells were subjected to transient transfection with the Sp1 expression plasmid, PₐSp1. Based on the expression of a β-galactosidase reporter gene, at least 80% of the B16F1 cells took up the exogenous DNA. The results of three independent experiments (Figure 1) showed Sp1 protein to be increased 2- to 3-fold compared to the empty vector controls at 36 h after the introduction of the Sp1 cDNA.

The murine cathepsin B gene is thought to contain three transcription start sites that give rise to alternative first exons that have been designated 1A, 1B and 1C (15). In order to investigate the effect of Sp1 on cathepsin B transcription in B16F1 cell, we designed 5’ primers to measure each of the three transcript types by reverse transcription-linked PCR. The 3’ primer corresponded to a common sequence across the junctions of exons 5 and 6. Figure 2 shows that introducing Sp1 into B16F1 cells increases messages containing exon 1A as determined by qualitative RT-PCR. In contrast, these same methods failed to detect messages containing exons 1B and 1C in cells transfected with either PₐSp1 or Pₐ0 (data not shown).
Cathepsin B activity and invasiveness of cells transfected with Sp1. Cathepsin B specific activity in cell lysates at 36 h posttransfection with either a human Sp1 cDNA (pPacSp1) or with the empty expression plasmid (pPac0). B16F1 cells were treated with a mixture of 10 µg of plasmid DNA and 10 µL of LIPOFECTAMINE reagent in 1 mL of serum-free medium. The experiment was repeated 5 times and the resulting cells were used as indicated in the motility and invasion assays shown in Figure 3.

Table I. Cathepsin B activity in B16F1 cells after transient transfection with the pPacSp1. Each value represents the mean±S.E.M. of three determinations of cathepsin B specific activity in cell lysates 36 h after transfection with either a human Sp1 cDNA (pPacSp1) or with the empty expression plasmid (pPac0). B16F1 cells were treated with a mixture of 10 µg of plasmid DNA and 10 µL of LIPOFECTAMINE reagent in 1 mL of serum-free medium. The experiment was repeated 5 times and the resulting cells were used as indicated in the motility and invasion assays shown in Figure 3.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>pPac0</th>
<th>pPacSp1</th>
<th>% control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility</td>
<td>3.1 ± 0.6</td>
<td>9.2 ± 0.5</td>
<td>300</td>
</tr>
<tr>
<td>Invasion 1</td>
<td>2.8 ± 0.5</td>
<td>8.0 ± 0.6</td>
<td>300</td>
</tr>
<tr>
<td>Invasion 2</td>
<td>3.3 ± 0.3</td>
<td>8.3 ± 0.3</td>
<td>250</td>
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<td>Invasion 3</td>
<td>2.3 ± 0.1</td>
<td>7.9 ± 0.5</td>
<td>340</td>
</tr>
<tr>
<td>Invasion 4</td>
<td>2.0 ± 0.1</td>
<td>10.5 ± 0.3</td>
<td>530</td>
</tr>
</tbody>
</table>

Discussion

The cathepsin B gene in mice is suggested to contain three transcription start sites that give rise to alternative first exons (15). The message containing exon 1A corresponds to the mouse cathepsin B cDNA that was originally cloned and sequenced by Chan and co-workers (14) The putative transcription start sites responsible for messages containing exons 1B and 1C were detected by 5' RACE in Kirsten virus-transformed BALB/3T3 cells. These were mapped within the cathepsin B gene at about 2.3-kbp and 80-bp upstream of exon 2, respectively, but the adjacent sequences were not evaluated in a functional assay of promoter activity (15). Competitive RT-PCR performed in this laboratory has
shown that messages containing exons 1B and 1C were at best present as minor components of the total cathepsin B mRNA in most normal mouse tissues and in a variety of murine tumors including the B16 melanomas (unpublished results). Our present result, which shows that Sp1 acts to primarily increase messages containing exon 1A in B16F1 cells, supports those findings.

A DNA fragment from the 5' end of the cathepsin B gene containing a portion of exon 1A and adjacent untranscribed sequences was previously found to function as a strong promoter in B16 melanoma cells (10). This fragment contained binding sites for the transcription factors Sp1 and Sp3. In addition, the levels of Sp1 and cathepsin B were much higher in the B16a melanoma compared to the less metastatic B1F1 variant, suggesting a role for Sp1 in elevated cathepsin B transcription in cancer (submitted for publication by Ahmed and Frankfater, 2004). Recently, a similar fragment from the 5' end of the human cathepsin B gene was found to contain binding sites for Sp1 and Ets1, and these factors were able to activate cathepsin B transcription in a human glioma (17). Levels of Sp1 are elevated in some transformed cells (18, 19), and Sp1 activation has been linked to cyclin A deregulation and cell growth in cancer (20). Interestingly, the human promoter can substitute for the mouse promoter to drive higher levels of gene transcription in B16a compared to B16F1 cells (10). Thus, the regulation of cathepsin B expression in murine and human tumors may be similar.

It is of interest to compare the effects of cathepsin B and Sp1 on the invasiveness of B16F1 cells in the Matrigel invasion assay. In a prior study, we were able to produce a 3.5- to 5-fold increase in cathepsin B activity 36 h after transient transfection with a human cathepsin B cDNA. This produced a nearly proportionate increase of 3- to 4-fold in invasiveness (8) that was inhibited by the methyl ester of CA-074, which specifically targets the intracellular form of cathepsin B. Here, the introduction of Sp1 into B16F1 cells caused a 2.5- to 5.3-fold increase in cathepsin B activity after 36 h and a 4.8- to 5.5-fold increase in the number of cells that traversed the Matrigel matrix. Although CA-074Me was again inhibitory, it failed to reduce invasiveness of Sp1-transfected cells to levels observed with control cells, suggesting either that an extracellular form of cathepsin B also contributes to matrix invasion or that other genes activated by Sp1 participate in matrix degradation. In this regard, Sp1 has been shown to be required for the constitutive or activated expression of the plasminogen activators and their receptors, some matrix metalloproteinases and cathepsins D and L (21-30).

In conclusion, Sp1 activates the expression of endogenous cathepsin B in murine B16 melanomas, resulting in an increase in the ability of the cells to invade a reconstituted extracellular matrix that can be attributed in part to cathepsin B.

References


Received May 4, 2004
Accepted June 28, 2004