Inhibition of Matrix Metalloproteinase-2 Expression and Bladder Carcinoma Metastasis by Halofuginone

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Abstract

Matrix metalloproteinase-2 (MMP-2) plays a critical role in tumor cell invasion and metastasis. Inhibitors of this enzyme effectively suppress tumor metastasis in experimental animals and are currently being tested in clinical trials. MMP-2 transcriptional regulation is a part of a delicate balance between the expression of various extracellular matrix (ECM) constituents and ECM degrading enzymes. Halofuginone, a low-molecular-weight quinazolinone alkaloid, is a potent inhibitor of collagen type α1 (I) gene expression and ECM deposition. We now report that expression of the MMP-2 gene by murine (MBT2-t50) and human (5637) bladder carcinoma cells is highly susceptible to inhibition by halofuginone. Fifty percent inhibition was obtained in the presence of as little as 50 ng/ml halofuginone. This inhibition is due to an effect of halofuginone on the activity of the MMP-2 promoter, as indicated by a pronounced suppression of chloramphenicol acetyltransferase activity driven by the MMP-2 promoter in transfected MBT2 cells. There was no effect on chloramphenicol acetyltransferase activity driven by SV40 promoter in these cells. Halofuginone-treated cells failed to invade through reconstituted basement-membrane (Matrigel) coated filters, in accordance with the inhibition of MMP-2 gene expression. A marked reduction (80–90%) in the lung colonization of MBT2 bladder carcinoma cells was obtained after the i.v. inoculation of halofuginone-treated cells as compared with the high metastatic activity exhibited by control untreated cells. Under the same conditions, there was almost no effect of halofuginone on the rate of MBT2 cell proliferation. These results indicate that the potent antimetastatic activity of halofuginone is due primarily to a transcriptional suppression of the MMP-2 gene, which results in a decreased enzymatic activity, matrix degradation, and tumor cell extravasation. This is the first description, to our knowledge, of a drug that inhibits experimental metastasis through the inhibition of MMP-2 at the transcriptional level. Combined with its known inhibitory effect on collagen synthesis and ECM deposition, halofuginone is expected to exert a profound antitumor effect by inhibiting both the primary tumor stromal support and metastatic spread.

Introduction

The invasive behavior of neoplastic cells and their ability to metastasize to distant sites are multistep processes that include detachment of the cells from the original tumor mass, attachment to ECM binding sites, degradation of ECMs, and migration into surrounding tissues (1–3). One of the rate-limiting steps in the metastatic cascade is the activity of MMPs degrading a variety of ECM proteins (2–6). A central role in this process is played by MMP-2 (M, 72,000 collagenase type IV), a zinc–dependent endopeptidase that cleaves primarily type IV collagen in the basement membrane. MMP-2 is subject to three levels of regulation including transcriptional control, proenzyme activation, and the inhibition by tissue inhibitors of MMPs (4, 5). Several compounds that inhibit MMP-2 enzymatic activity suppress tumor cell metastasis in experimental animals (7–10). A more specific and efficient approach can be achieved through a transcriptional inhibition of the MMP-2 gene. Development of novel inhibitors that suppress MMP-2 gene expression and, hence, enzymatic activity is, therefore, regarded as a rational approach to metastatic disease therapy.

MMP-2 transcriptional regulation is a part of a delicate balance between the biosynthesis of different ECM components and the expression of various ECM degrading enzymes. Common regulatory pathways for ECM component production, deposition, and degradation were described, mainly through TGF-β1-mediated transcriptional effects (11, 12). TGF-β1 is a pluripotent regulator of a variety of cellular activities including cell growth, differentiation, and synthesis of ECM constituents (11–13). In fact, TGF-β1 promotes expression of genes encoding several types of collagen (i.e., I, III, and V) and collagen-degrading enzymes such as MMP-2 (14–20), which suggests a...
possible common regulatory element in the transcriptional control of ECM proteins (i.e., collagen type I) and MMP-2.

In previous studies, we have demonstrated that halofuginone (Fig. 1), a low-molecular-weight quinazolinone-derived alkaloid isolated from the plant *Dichroa Febrifuga* and widely used for over 20 years as a coccidiostat in chickens and turkeys (21), suppresses collagen type α1 (I) gene expression and ECM deposition (21–25). Specific inhibition of collagen type I synthesis was demonstrated in a broad range of cell types of chicken, mouse, rat, and human origin, both in vitro and in experimental animals (21–23). Halofuginone was also shown to inhibit TGF-β-stimulated collagen α1 (I) synthesis by human skin fibroblasts (23). Moreover, exposure to halofuginone was found to inhibit deposition of ECM by vascular smooth muscle (24) and kidney mesangial (25) cells.

With this in mind, we tested whether halofuginone affects the expression of the MMP-2 gene and its involvement in tumor cell invasion and metastasis. Using a highly metastatic variant of the MBT2 murine bladder carcinoma cell line (26)—MBT2-t50 (27, 28)—we demonstrated a strong inhibitory effect of halofuginone on the MMP-2 promoter activity. This inhibition resulted in a marked decrease of both ECM invasion, using the Matrigel invasion assay, and lung colonization by the MBT2-t50 bladder carcinoma cells. The present results are consistent with the hypothesis of a possible common transcriptional regulation of ECM components and ECM degrading enzymes.

### Materials and Methods

#### Cell Culture.
A highly metastatic variant (MBT2-t50) of the MBT2 murine bladder carcinoma cell line was kindly provided by Dr. O. Medalia (Sackler Medical School, Tel-Aviv University; Refs. 27, 28). A human bladder carcinoma cell line, 5637, was obtained from the American Type Culture Collection. The cells were maintained in DMEM (4.5 g glucose/liter) supplemented with 10% FCS, L-glutamine, and antibiotics (Beit Haemek Biological Industries, Israel) at 37°C in 5% CO2 humidified incubator. Cells were subcultured twice a week with trypsin/EDTA solution (saline containing 0.05% trypsin, 0.01 M sodium phosphate (pH 7.4) and 0.02% EDTA).

#### Zymography.
Cells were seeded into 35-mm culture plates (2.5 × 10^5 cells/plate) and maintained for 24 h in DMEM supplemented with 10% FCS. Subconfluent cell cultures were incubated for 24 h in the absence or presence of increasing concentrations (10–200 ng/ml) of halofuginone (Roussel-Uclaf, Paris, France; dissolved in H2O/10% ethanol) in serum-free DMEM (2 ml/plate), and aliquots of the resultant conditioned medium were analyzed for gelatinolytic activity. Conditioned medium of human melanoma SB-2 cells expressing both MMP-2 and MMP-9 (29) was used as a positive control. MMP-2 activity was determined on gelatin impregnated (1 mg/ml; Difco, Detroit, MI) SDS-8% polyacrylamide gels, as described previously (29, 30). Briefly, samples of the culture media, normalized for equal cell protein, were separated on the substrate-impregnated gels under nonreducing conditions, followed by 30-min incubation in 2.5% Triton X-100 (Sigma, St. Louis, MO). The gels were then incubated for 16 h at 37°C in 50 mM Tris, 0.2 M NaCl, 5 mM CaCl2, 0.02% Brij 35 (w/v) at pH 7.6. At the end of the incubation period, the gels were stained with 0.5% Coomassie Blue G 250 (Bio-Rad, Richmond, CA) in methanol/acetic acid/H2O (30:10:60). The intensity of the various bands was determined by computerized densitometer (Molecular Dynamics type 300A).

#### Transfection and CAT Assay.
Using the lipofectin protocol (Life Technologies, Inc., Gaithersburg, MD), MBT2-t50 cells (5 × 10^5 cells/10 cm dish) were transfected with 2.5 μg of the CAT expression vector with or without MMP-2 promoter sequence (pCAT: promoter, or pCAT-basic: no promoter, respectively), or with a control plasmid with SV40 promoter (Promega, Madison, WI). The human MMP-2 promoter region spanning nucleotides (−390 to +290; Ref. 31) was ligated upstream of the basic or enhanced CAT expression vector and transfected to the MBT2-t50 cells as described above (29). The MMP-2 promoter segment was generated by PCR using primers encompassing both ends of the domain and sticky ends of HindIII and XbaI, respectively, as described previously (29). The promoter construct was sequenced and found to be identical to the authentic sequence presented in Ref. 31. Four h after transfection, the cells were exposed to increasing concentrations (50–200 ng/ml) of halofuginone. After 48 h, cell extracts were prepared, normalized for protein concentration, and assayed for CAT activity as described previously (29, 32). The CAT assay was quantified in triplicates by densitometry.

#### Matrigel Invasion Assay.
Blind-well chemotaxis chambers with filters 13 mm in diameter were used. Polyvinylpyrrolidone-free polycarbonate filters with pores 8 μm in size (Costar Scientific, Cambridge MA) were coated with basement membrane Matrigel (25 μg/filter) as described previously (29, 33). Cells (2 × 10^5) suspended in DMEM containing 0.1% BSA were added to the upper chamber in the absence or presence of increasing concentrations (10–100 ng/ml) of halofuginone. Medium conditioned by 3T3 fibroblasts was applied as a chemoattractant and placed in the lower compartment of the Boyden chamber (29, 33). Cells were assayed at 37°C in 5% CO2 for 6 h. More than 90% of the cells attached to the filter after a 2-h incubation. The number of cells attached to the filter under these conditions was counted by a microscopic examination of five microscopic fields, and the percentage of attached cells/filter was calculated. Halofuginone had no effect on cell adhesion to Matrigel-coated filters. At the end of the incubation, the cells on the upper surface of the filter were removed by wiping with a cotton swab, and cells on the lower surface of the filter were stained with Diff-Quick stain (American Scientific Products, McGaw Park, IL). Cells in different areas of the lower surface were counted, and each assay was done in triplicate. For chemotaxis studies, filters were coated with collagen type IV alone (5 μg/filter) to promote cell adhesion. This amount of collagen...
Experimental Metastasis. Four h before cell harvesting, halofuginone (0.5 and 2.5 μg/ml) was added to the growth medium of the MBT2-t50 cells. Subconfluent cells were harvested by trypsin/EDTA for 3 min at 37°C. The cells were collected, washed in complete medium, centrifuged at 1000 rpm for 10 min, and resuspended in PBS into a single-cell suspension (7.5 × 10⁶ cells/ml). Halofuginone (0.5 and 2.5 μg/ml) was re-added to the cell suspension followed by the injection of 3 × 10⁴ cells into the lateral tail vein of 7–8-week-old C3H male mice (Harlan Laboratories, Jerusalem, Israel). The injection schedule alternated between animals of the control group injected with untreated cells and animals of the experimental group, to minimize experimental variance, especially time. Animals were killed 11 days after injection, the lungs were resected, and metastatic nodules on the lung surface under a dissecting microscope (8, 34).

Results

Expression of MMP-2 Gelatinolytic Activity by Cells Treated with Halofuginone. We investigated the effect of halofuginone on MMP-2 activity expressed by MBT2-t50 cells. For this purpose, subconfluent cultures were treated (24 h) with increasing concentrations (10–200 ng/ml) of halofuginone in serum-free medium. Aliquots of the conditioned medium were applied onto gelatin-embedded SDS-polyacrylamide gels. The zymogram shown in Fig. 2A demonstrates that the gelatinolytic activity of the MMP-2 enzyme was markedly reduced by halofuginone. Densitometric analysis of the zymogram revealed a 50% inhibition of MMP-2 activity already in cells pretreated with as little as 10 ng/ml of halofuginone. An almost complete inhibition of MMP-2 activity was exerted by 100–200 ng/ml halofuginone (Fig. 2A). Similar results were obtained with 5637 human bladder carcinoma cells (Fig. 2B). The addition of halofuginone (100–200 ng/ml) to the zymogram reaction buffer had no effect on MMP-2 activity, which indicates that the drug does not exert a direct inhibitory effect on the MMP-2 enzyme itself.

Effect of Halofuginone on MMP-2 Promoter Activity. We next examined whether the inhibitory effect of halofuginone on collagenase activity is due to inhibition of MMP-2 transcription. For this purpose, the MMP-2 promoter (−390 to +291) was linked upstream of the CAT reporter gene and the construct was transfected into MBT2-t50 murine bladder carcinoma cells. Control cells were transfected with either the CAT reporter gene driven by SV40 promoter (pCAT-SV40) or by the basic CAT expression vector (pCAT-basic). The cells were then exposed (48 h, 37°C) to increasing concentrations (50–200 ng/ml) of halofuginone in complete medium, and equivalent amounts of cell extracts were tested for CAT activity (Fig. 3). CAT activity driven by the MMP-2 promoter was inhibited by 50% in the presence of 100 ng/ml halofuginone (Fig. 3). About 80% inhibition of MMP-2 promoter activity was exerted by 200 ng/ml halofuginone (Fig. 3). CAT expression driven by SV40 promoter was not affected by 200 ng/ml halofuginone and served as an internal control for transfection efficiency (not shown). CAT activity was not detected in cells transfected with the basic CAT expression vector (Fig. 3). These results indicate that halofuginone suppresses transcriptional activity of the MMP-2 promoter in a dose-dependent manner, resulting in a profound decrease in the MMP-2 enzyme expressed by MBT2-t50 cells.

Effect of Halofuginone on MBT2-t50 Cell Invasion through Matrigel-coated Filters. MMP-2 was previously shown to play a central role in basement membrane invasion both in vitro and in vivo (4, 5). We, therefore, tested the effect of halofuginone on MBT2-t50 cell invasion using the Matrigel invasion assay. The presence of as little as 10 ng/ml halofuginone during the chemo invasion assay resulted in a 50% inhibition of the ability of MBT2-t50 cells to invade through Matrigel-coated filters (Fig. 4). Cells treated with 50 ng/ml halofuginone...
failed to invade the Matrigel layer. The MBT2-t50 bladder carcinoma cells readily traversed through filters coated with 5 μg collagen IV, to a similar extent in the absence or presence of 50 ng/ml halofuginone (not shown), which indicated that halofuginone had no effect on cell viability and motility.

Effect of Halofuginone on Experimental Metastasis. Because halofuginone efficiently inhibited MMP-2 gene expression and MBT2-t50 cell migration through Matrigel, we tested its effect on experimental metastasis in vivo. For this purpose, MBT2-t50 cells were pretreated in culture (4 h, 37°C) with halofuginone (0.5 and 2.5 μg/ml), dissociated with trypsin/EDTA, and injected into the tail vein of C3H mice (0.3 × 10⁵ cells per mouse) together with 0.5 or 2.5 μg/ml halofuginone. Eleven days afterward, the mice were killed, and their lungs were evaluated for the number of surface metastatic nodules. A profound antimetastatic effect of halofuginone (80–90% inhibition) was observed in animals injected with cells that were pretreated with halofuginone (Fig. 5A). The morphological appearance of representative MBT2 colonized lungs derived from halofuginone-treated and untreated mice is illustrated in Fig. 5B.

Discussion

We have demonstrated a significant inhibition of experimental metastasis by MBT2-t50 cells that were treated with halofuginone, a low-molecular-weight alkaloid compound known to specifically inhibit collagen type α1 (I) but not collagen type II, III, or X gene expression (Refs. 21, 22, 35 and our unpublished results). Halofuginone was shown to inhibit collagen synthesis and ECM deposition in a variety of cell types (21–25), resulting in amelioration of a number of fibrotic disorders (23, 36, 37). Halofuginone also exerted an antiproliferative effect on various cell types, although this effect may or may not be related to its inhibition of collagen type α1 (I) gene

5 Unpublished observations.
expression. The profound inhibition of experimental metastasis by halofuginone is a novel unexpected feature of this compound, apparently unrelated to its well-characterized effect on collagen \( \alpha_1 \) (I) synthesis. In the present study, tumor cells were exposed to halofuginone for a short period and tested for their ability to invade a natural basement membrane-like material (Matrigel) as well as to exit from the blood circulation and metastasize into the lung tissue. It is well recognized that only cells that are capable of extravasation within the first 2–3 h after inoculation do form metastatic nodules, whereas cells that remain in the blood stream for a longer time fail to survive (38). Hence, it appears that the antimetastatic activity of halofuginone cannot be attributed to its inhibition of collagen synthesis and cell proliferation but rather to a short-term effect on parameters related to cell invasion. Relatively high concentrations (0.5–2.5 \( \mu g/ml \)) of halofuginone were applied in the in vivo versus the in vitro experiments. This is due primarily to the short half-life of the drug in the circulation.\(^5\)

A critical step in the extravasation of blood-borne cells is the degradation of the subendothelial ECM (1–6). Collagenase type IV (MMP-2) is regarded as a key enzyme involved in tumor invasion and metastasis, as indicated by the antimetastatic effects of several collagenase inhibitors, some of which are already being applied in clinical trials (7–10). We now report that halofuginone inhibits MMP-2 expression, resulting in an almost complete inhibition of gelatinolytic activity in the medium of cells grown in the presence of as little as 100 ng/ml halofuginone, as demonstrated by zymogram gels. This inhibition was not restricted to MBT2-t50 murine bladder carcinoma cells, but was also demonstrated in cells of human (5637 bladder carcinoma) and bovine (aortic endothelium) origin (data not shown). Using a CAT reporter gene driven by the MMP-2 promoter, we have demonstrated that this activity is due to an inhibitory effect of halofuginone on the MMP-2 promoter activity. In fact, it was previously suggested that the MMP-2 and collagen type \( \alpha_1 \) (I) genes may share common transcriptional regulatory elements (17). For example, it was found that TGF-\( \beta 1 \) increases both MMP-2 and collagen type I expression levels, possibly through modulation of common transcription factor(s) (14, 15, 17–20). A coordinated, TGF-\( \beta \) mediated regulation of MMP-2 and MMP-2-susceptible ECM constituents such as type I collagen and fibronectin has been reported previously (17), which suggests an important role for MMP-2 in the establishment of ECMs during embryogenesis, remodeling, and wound healing. Recently, it was reported that the chemotherapeutic drug bleomycin, known to activate collagen type I gene, also markedly increases MMP-2 gene expression (39). Our finding—that halofuginone, a potent inhibitor of collagen type I gene expression, also inhibits MMP-2 transcription—is consistent with a common regulation of both genes. In support of such a proposed mode of action is the observation that halofuginone inhibits TGF-\( \beta \)-induced collagen synthesis in human skin fibroblasts (23). The molecular mechanism by which TGF-\( \beta \) exerts its transcriptional effects is still poorly understood. Several transcription factors were proposed as targets of TGF-\( \beta \)-mediated gene regulation. Computerized analysis of the promoter sequences of MMP-2 and collagen \( \alpha_1 \) (I) using the MathInspector program (40) revealed potential binding sites for at least six of these target transcription factors (i.e., AP1, CREB, SP1, Oct,

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**Fig. 5** Effect of halofuginone on lung colonization by MBT2-t50 murine bladder carcinoma cells. Subconfluent MBT2-t50 cells were treated (4 h, 37°C) with 0.5 \( \mu g/ml \) and 2.5 \( \mu g/ml \) halofuginone, dissociated with trypsin/EDTA, washed, and suspended in PBS as described in “Materials and Methods.” Halofuginone was added to the cell suspension to a final concentration of 0.5 and 2.5 \( \mu g/ml \), and 0.4 ml containing \( 3 \times 10^4 \) cells was injected into the lateral tail vein of each C3H syngeneic mouse. Control mice were injected with untreated MBT2-t50 cells. Mice were killed 11 days later, and the lungs were removed and fixed in Bouin’s solution. A, the number of metastatic lung nodules per mouse (mean ± SD, \( n = 5 \)). Statistical difference between control and each of the experimental groups is highly significant (\( P < 0.0001 \)); B, gross morphological appearance of representative lung lobes taken from control (left) and halofuginone-treated (right) mice. The experiment was performed three times with similar results.
CTF/NF-I, and SRF/TEF) in each of these promoters. Clearly, only a few of these binding sites may actually take part in the proposed coordinated regulation of the MMP-2 and collagen I genes.

We have recently found an inhibitory effect of halofuginone on the growth of a chemically induced murine bladder tumor and a tumor derived from s.c. injected bladder carcinoma cells (41). It was attributed to a direct inhibition of cell proliferation and to suppression by halofuginone of ECM deposition and, hence, the stromal support involved in tumor progression (42). In a recent experiment, we observed a profound decrease in the level of H19 in two types of human bladder cancer cells treated with halofuginone. H19 is regarded as a reliable tumor marker, particularly in bladder cancer (43–45), which further emphasizes the potential effectiveness of halofuginone in inhibiting the progression of bladder tumors. We have also demonstrated that halofuginone inhibits vascular tube formation and neovascularization both in vitro (collagen-embedded rat aortic ring assay) and in vivo (mouse corneal micropocket assay). This effect of halofuginone may be attributed to the observed inhibition of MMP-2 transcription because this enzyme is known to play an important role in endothelial cell invasion and matrix degradation involved in the angiogenic process (46), in a manner similar to its involvement in tumor cell invasion and metastasis. Because collagen type I forms a scaffold assisting directional vascular tube formation, the inhibitory effect of halofuginone on angiogenesis can be attributed to the suppression of both collagen I synthesis and MMP-2 activity.

It should be noted that the effects of halofuginone on tumor growth and angiogenesis were reversible. Moreover, no toxic effects were observed in mice receiving oral halofuginone for over 6 months. These features make halofuginone a likely candidate for additional studies in animal models of tumor progression, toward clinical trials in human. Altogether, our results suggest that halofuginone may exert a profound antinecrotic effect through a combined action on several critical stages in tumor progression such as cell proliferation, angiogenesis, stromal support, and metastatic spread.

References


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