Inhibition of Bladder Carcinoma Angiogenesis, Stromal Support, and Tumor Growth by Halofuginone

Michael Elkin, Ilana Ariel, Hua-Quan Miao, Arnon Nagler, Mark Pines, Nathan de-Groot, Avraham Hochberg, and Israel Vlodavsky

ABSTRACT

We have previously demonstrated that halofuginone, a widely used alkaloid coccoidiostat, is a potent inhibitor of collagen α1(I) and matrix metalloproteinase 2 gene expression. Halofuginone also suppresses extracellular matrix deposition and cell proliferation. We investigated the effect of halofuginone on transplantable and chemically induced mouse bladder carcinoma. In both systems, oral administration of halofuginone resulted in a profound anticancerous effect, even when the treatment was initiated at advanced stages of tumor development. Although halofuginone failed to prevent proliferative preneoplastic alterations in the bladder epithelium, it inhibited further progression of the chemically induced tumor into a malignant invasive stage. Histological examination and in situ analysis of the tumor tissue revealed a marked decrease in blood vessel density and in both collagen α1(I) and H19 gene expression. H19 is regarded as an early marker of bladder carcinoma. The antiangiogenic effect of halofuginone was also demonstrated by inhibition of microvessel formation in vitro. We attribute the profound antitumoral effect of halofuginone to its combined inhibition of the tumor stromal support, vascularization, invasiveness, and cell proliferation.

INTRODUCTION

Solid tumors are composed of two distinct but interdependent compartments, the malignant cells themselves and the ECM, which provides stromal support for the growing tumor. The major stromal components include structural proteins such as interstitial collagens (types I and III and, to a lesser extent, type V), fibrin, fibronectins in several isoforms, tenascin, elastin, and sulfated proteoglycans (1). In fact, all solid tumors, regardless of their type or cellular origin, require stroma if they are to grow beyond minimal size (1). Stromata provide the vascular supply that tumors require for obtaining nutrients, gas exchange, and waste disposal (1–3). The ECM plays an active and complex role in regulating gene expression and behavior of tumor cells that contact it, influencing their development, migration, proliferation, and metabolic functions (1, 4). Hence, any treatment that destroys stroma or interferes with its generation would be expected to have profound consequences on tumor growth and survival (1). On the other hand, stroma, and especially basement membrane, may limit tumor invasion into local host tissue, a process regarded as the hallmark of malignant neoplasia (5). One of the rate-limiting steps in basement membrane degradation is the activity of MMP-2 and MMP-9, zinc-dependent endopeptidases that cleave primarily type IV collagen, the most prominent protein in basement membrane (5–9). There is a substantial body of evidence that confirms a linkage between MMPs and the malignant phenotype in tumors of different origins, including bladder carcinoma (5–11). MMPs, in a manner similar to their involvement in tumor cell invasion, also play an important role in endothelial cell sprouting, the initial phase of tumor-associated angiogenesis (12–17). MMPs are, therefore, regarded as key enzymes in primary tumor progression and metastatic spread.

We have previously demonstrated that halofuginone, a low molecular weight quinazolinone alkaloid (495 Da, structure presented in the Merck Index) isolated from the plant dichroa febrifuga and widely used as a coccoidiostat in chickens and turkeys (18), is a potent inhibitor of collagen type α1(I) gene expression and ECM deposition (19–22). 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 (20–22). Recently, we have found that halofuginone also suppresses transcription of the MMP-2 gene, associated with a marked decrease in ECM invasion in vitro and lung colonization by bladder carcinoma cells (23).

In the present study, we investigated the inhibitory effect of halofuginone on tumor progression in two models of mouse bladder carcinoma: (a) tumor formed by s.c. transplantation of MBT2-t50 murine bladder carcinoma cells into syngenic mice; and (b) bladder carcinoma induced by BBN, a bladder-specific carcinogen (24–27). A strong anticancerous effect of halofuginone was obtained in both experimental models, even when the halofuginone treatment was initiated at relatively advanced stages of tumor development. In addition, a profound decrease in the level of H19 gene expression was observed in response to halofuginone treatment, further emphasizing its effectiveness in inhibiting the progression of bladder tumors. H19 is regarded an early marker of tumorigenesis in a variety of tissues, including bladder (27–29). We attribute the antitumoral effect of halofuginone to its action on several critical steps in primary tumor progression, such as angiogenesis, stromal support and cell proliferation.

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, Tel-Aviv, Israel; Refs. 30 and 31). The cells were maintained in DMEM (4.5 g of glucose/liter) supplemented with 10% FCS, L-glutamine, and antibiotics (Biological Industries, Beit-Haemek, Israel) at 37 °C in an 8% CO2 humidified incubator. Cells were seeded in DMEM containing 10% FCS at a density of 3 × 104 cells/16-mm well of a 24-well plate, in quadruplicate. The medium was replaced 24 h after seeding, and the cells were cultured for 7 days in the absence or presence of different concentrations of halofuginone, ranging from 2.5–30 ng/ml. Halofuginone was kindly provided by Roussel-Uclaf (Paris, France) and dissolved in H2O/10% ethanol. On each day after seeding,
cells of four wells were dissociated with trypsin/EDTA and counted with a Coulter counter (Coulter Electronics Ltd.).

**Inoculation of MBT2-t50 Cells.** MBT2-t50 cells were dissociated into a single cell suspension (10^6 cells/ml), and 2 × 10^5 cells were injected via a 25-gauge needle into the s.c. space on the dorsa of C3H/He male mice (Harlan Laboratories, Jerusalem, Israel), 6 weeks of age. The mice were divided into four groups receiving different amounts of halofuginone in the diet starting on the day of tumor cell inoculation or on day 10 of the experiment, when the tumors became palpable. Twenty days after cell injection, the mice were sacrificed and the s.c. tumors were measured by calipers and removed. Tumor size was estimated using the formula V = 0.5 × LW^2, in which L is tumor length and W is tumor width. Samples of tumor tissue were fixed in 4% buffered formaldehyde solution and processed for histological examination.

For vessel density analysis, 5-μm thick sections from paraffin-embedded tumor tissue were stained with H&E. Vascular structures were recognized as luminal or slit-like structures that occasionally contained blood cells within them and were delineated by flattened endothelial cells, as described by Takeshi et al. (17). The microvessel density was determined in tumor areas with the highest vascularization. Individual vessels were counted on ×200 and ×400 microscopic fields (0.785 mm² and 0.196 mm², respectively). A total of six fields/tumor (two sections × three fields) were analyzed, and the mean value ± SD was determined.

**In Vitro Angiogenesis.** Type I collagen was prepared from the tail tendons of adult Sprague Dawley rats (32). The collagen matrix gel was obtained by simultaneously raising the pH and ionic strength of the collagen solution (33). Thoracic aortas were obtained from Sprague Dawley rats, 2 months of age. The fibroadipose tissue was carefully removed under a dissecting microscope, and aortic rings were sectioned (1-mm long) and placed on top of 0.2 ml of collagen gel in 16-mm culture wells (34). Collagen solution (0.4 ml) was carefully poured on top of the ring. After the gel was formed, 0.4 ml of serum-free, Endothelial Growth Medium (Life Technologies, Inc.) was added and replaced every other day by fresh medium containing the indicated concentrations of halofuginone. Microvessel outgrowth was visualized by phase microscopy, and the number of capillary vessels was determined on days 8–10.

**Effect of Halofuginone on BBN-induced Bladder Tumorigenesis.** C3H/He male mice (Harlan Laboratories), 6 weeks of age, were divided into five groups, 6–10 mice/group. BBN (Tokyo Kasei Co., Tokyo, Japan) was dissolved in tap water to a concentration of 0.05% and supplied to all groups during the first 10 weeks of the experiment. Thereafter, the mice received tap water without BBN. Halofuginone was administered in the diet (5 mg/kg) starting on weeks 1, 6, 11, and 20 (groups 1, 2, 3, and 4, respectively). Group 5 (control group) was not treated with halofuginone. Mice of experimental groups 1–3 and control group 5 were sacrificed on week 26, and mice of experimental group 4 were sacrificed on week 29. The experimental design is presented in Fig. 5a. The bladders of all mice were removed, and 4% buffered formaldehyde solution was infused into the lumen. The bladders were then fixed in 4% buffered formaldehyde for 24 h, dissected along the mid-sagittal plane, and processed for histological and in situ hybridization with H19-specific riboprobe.

**In Situ Hybridization.** In situ hybridization for collagen α1(I) mRNA was performed as described previously (35, 36). Briefly, tissue sections were deparaffinized in xylene, rehydrated through a graded series of ethanol solutions, rinsed in distilled water (5 min), and incubated in 2 × SSC at 70°C for

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Fig. 1. Effect of halofuginone on MBT2-t50 tumor development. The s.c. dorsa of C3H/He mice were implanted with MBT2-t50 cells (2 × 10^5 in 0.2 ml of PBS). Mice were divided into four groups. Group 1 (n = 9) received normal diet without halofuginone (control). Group 2 (n = 11) received halofuginone-containing diet (5 mg/kg food) throughout the experiment. Group 3 (n = 9) received halofuginone-containing diet (5 mg/kg) starting on day 10 after cell inoculation, when the tumors became palpable; group 4 (n = 6) received a diet containing twice the amount of halofuginone (10 mg/kg). On day 20, tumors were measured by calipers, excised, fixed in 4% buffered formaldehyde solution, and processed for histological examination. a, mean and SE of tumor volume (width^2 × length × 0.50) of treated versus untreated mice; P < 0.0001; One-way ANOVA. b, macroscopic appearance of MBT2-t50 s.c. tumors excised from mice receiving normal diet (I), or diet containing 5 mg of halofuginone (II) and 10 mg of halofuginone (III)/kg food. c and d, histological analysis of H&E-stained sections of tumor tissue derived from untreated (c) and halofuginone (5 mg/kg)-treated (d) mice, ×200. Arrows, blood vessels.
The sections were then rinsed in distilled water and treated with Pronase [0.125 mg/ml in 50 mM Tris-HCl, and 5 mM EDTA (pH 7.5)] for 10 min. After digestion, the slides were rinsed with distilled water, postfixed in 10% formalin in PBS, blocked in 0.2% glycine, rinsed in distilled water, rapidly dehydrated through graded ethanol solutions, and air dried for several hours. Before hybridization, the 1600-bp rat collagen α1(I) insert was cut out from the original plasmid (pUC18) and inserted into pSafyre. The sections were then hybridized with digoxigen-labeled collagen α1(I) probe (35, 36). *In situ* hybridization for H19 mRNA was performed using an ~400 bp 35S-labeled antisense RNA probe, generated by *in vitro* transcription of plasmid subclone of murine H19 [kindly provided by Dr. S. M. Tilghman (Princeton University, Princeton, NJ)] by T3 polymerase (26). A sense RNA probe generated by T7 polymerase was used as control. The procedure of the *in situ* hybridization was carried out as described previously (26).

**RESULTS**

**Inhibition of MBT2-t50 Bladder Carcinoma Progression and Collagen α1(I) Expression by Halofuginone.** The inhibitory effect of halofuginone on collagen α1(I) (18–22) and MMP-2 gene expression (23), as well as its antiproliferative effect (21, 22), led us to investigate the anticancerous activity of halofuginone and its dependence on the dose and time of administration. For this purpose, C3H/He mice were injected (s.c.) with MBT2-t50 bladder carcinoma cells (2 × 10⁵ cells/mouse) and divided into four groups. Group 1 (control group) received a normal diet without halofuginone, whereas group 2 was fed with a halofuginone-containing diet (5 mg/kg food), starting from the day of cell inoculation. Mice of group 3 were maintained on a normal diet (without halofuginone) until the tumors reached a size of about 0.3 cm in diameter (day 10). The mice were then fed with halofuginone-containing diet (5 mg/kg). Group 4 received a diet containing twice as much halofuginone (10 mg/kg), starting from the day of cell inoculation. Twenty days after initiation of the experiment, tumor volumes were measured. A significant inhibition (60–70%; P < 0.0001) of tumor growth was observed in mice that were fed with halofuginone (5 mg/kg) from day 0 (group 2), as well as in mice that received halofuginone starting on day 10 (group 3), when palpable tumors were detected (Fig. 1). Similar results were obtained when 30 min. The sections were then rinsed in distilled water and treated with Pronase [0.125 mg/ml in 50 mM Tris-HCl, and 5 mM EDTA (pH 7.5)] for 10 min. After digestion, the slides were rinsed with distilled water, postfixed in 10% formalin in PBS, blocked in 0.2% glycine, rinsed in distilled water, rapidly dehydrated through graded ethanol solutions, and air dried for several hours. Before hybridization, the 1600-bp rat collagen α1(I) insert was cut out from the original plasmid (pUC18) and inserted into pSafyre. The sections were then hybridized with digoxigen-labeled collagen α1(I) probe (35, 36). *In situ* hybridization for H19 mRNA was performed using an ~400 bp 35S-labeled antisense RNA probe, generated by *in vitro* transcription of plasmid subclone of murine H19 [kindly provided by Dr. S. M. Tilghman (Princeton University, Princeton, NJ)] by T3 polymerase (26). A sense RNA probe generated by T7 polymerase was used as control. The procedure of the *in situ* hybridization was carried out as described previously (26).
halofuginone was administered daily (1 μg/day/mouse) by i.p. injections (data not shown). Whereas the mean tumor size ± SE in halofuginone-treated mice was 0.87 ± 0.26 cm³ and 0.98 ± 0.17 cm³ (groups 2 and 3, respectively), control mice (group 1) maintained on a normal diet developed large tumors with an average size of 2.76 ± 0.32 cm³ (Fig. 1a). An even more pronounced antitumoral effect was observed in mice fed with a 2-fold higher amount of halofuginone (10 mg/kg diet; group 4), yielding at least a 10-fold reduction in tumor size (0.24 ± 0.21 cm³, P < 0.0001, one way ANOVA; Fig. 1a). At the end of the experiment (day 20), the mice were sacrificed and the tumors were excised and photographed (Fig. 1b). A marked inhibition of tumor development was noted. A sample of the tumor tissue was processed for histological examination (Fig. 1, c and d) and in situ hybridization (Fig. 2). In situ analysis of collagen α1(I) gene expression in tissue sections derived from untreated mice revealed significant levels of collagen α1(I) gene transcripts, primarily in the stroma (tumor capsule) surrounding the tumor mass (Fig. 2a). Halofuginone treatment resulted in a profound decrease in collagen α1(I) gene expressed by the stromal cells (Fig. 2b).

Effect of Halofuginone on Microvascular Density in Vivo and Vascular Tube Formation in Vitro. To examine the effect of halofuginone on neovascularization in MBT2-t50 s.c. tumors, we compared microvascular density in H&E-stained sections of tumor tissue obtained from halofuginone-treated and untreated mice. Representative microphotographs of areas with the highest density of microvessels in tumor tissue sections derived from both control and experimental groups are shown in Fig. 1, c and d, respectively. In the control (untreated) group, the mean microvessel count was 7.6 ± 1.7/200/microscopic field and 4 ± 0.4/×400 field. In the experimental group receiving halofuginone in the diet, the corresponding values were 3.2 ± 1.0/×200 field and 2.2 ± 0.6/×400 field, respectively. Student’s t test revealed the difference between the groups to be statistically significant (P = 0.0043 and 0.0015, respectively). This observation led us to investigate the effect of halofuginone on microvessel formation in vitro, using rat aortic rings embedded in a collagen gel (34). Branching microvessels forming a capillary network of tubes and loops were developed at the periphery of untreated aortic rings, starting on days 4–5 and reaching a maximal degree of sprouting on days 8–10 (Fig. 3a). In contrast, microvessel formation was completely inhibited in the presence of halofuginone. Under this condition, single cells were migrating out of the aortic ring, but failed to align into microvessel tubes (Fig. 3b). A quantitative analysis of the number of outgrowing microvessels revealed a 50% inhibition at 25 ng/ml halofuginone and an almost complete inhibition of vascular tube formation at 50–100 ng/ml halofuginone.

Antiproliferative Effect of Halofuginone. The antitumoral effect of halofuginone may be attributed, apart from suppression of collagen synthesis, stromal support and angiogenesis, to a direct inhibition of tumor cell proliferation. As shown in Fig. 4, the culturing of MBT2-t50 bladder carcinoma cells in the presence of <10 ng/ml halofuginone did not result in detectable inhibition of cell proliferation. An almost complete inhibition of cell growth was obtained in the presence of 30 ng/ml halofuginone (Fig. 4). Measurements of the plasma levels of halofuginone in mice fed during 14 days with halofuginone-containing diet (5 mg/kg) yielded a concentration of 2 ng/ml, suggesting that the inhibition of tumor growth by halofuginone cannot be attributed to its antiproliferative activity.

Effect of Halofuginone on BBN-induced Bladder Carcinoma Progression and H19 Expression. Urinary bladder carcinoma is readily induced by the chemical carcinogen BBN, both in rats and mice (24–26). Following administration of BBN in the drinking water, the bladder epithelium sequentially undergoes mild hyperplasia (week 5 of BBN administration), severe hyperplasia, and dysplasia (week 10), leading to invasive carcinoma at week 20 with progression to muscular invasion at weeks 26–28 in all cases that developed cancer at that stage (25, 26). We applied this model of bladder-specific carcinogenesis to investigate the effect of halofuginone on the induction and progression of bladder carcinoma in mice. For this purpose, BBN (0.05%) was given to C3H mice in the drinking water during the first 10 weeks. Oral halofuginone (5 mg/kg food) was administered at different stages (week 1, normal epithelium; week 6, early hyperplastic changes; week 11, severe hyperplasia and dysplastic changes; and week 20, bladder neoplasia) of tumor progression. Control mice were treated with BBN in the same manner and were fed with normal diet. A diagram describing the experimental design is presented in Fig. 5a. Mice were sacrificed at week 26 (or week 29 in the case of mice treated with halofuginone starting on week 20), and the bladders were removed, fixed, and subjected to histological examination. The antitumoral effect of halofuginone was clearly demonstrated by macroscopic examination of the bladders. Five of nine bladders derived from control mice, receiving no halofuginone, exhibited well-established tumors, 3–8 mm² in size, as compared with normal-sized bladders without visible tumors in mice from each of the halofuginone-treated groups that were sacrificed at week 26 (Fig. 5, b and c). Microscopically, tumors invading the full-thickness of the muscular layer, causing extensive destruction of the bladder wall were seen in seven of nine mice that were not treated with halofuginone (Fig. 5d and Table 1). In the remaining two mice, 10–15-layer thick hyperplasia of the mucosa, as well as nodular hyperplasia and dysplastic changes, was noted in the bladder mucosa (Table 1). In contrast, regardless of the time in which the halofuginone treatment was initiated (weeks 1, 6, and 11), invasive carcinoma could not be detected in any mouse, except for 2 of 23 bladders, in which superficial carcinoma invading only the submucosa was noted (Table 1). In about 80% of the mice, tumor progression reached the stage of mild hyperplasia (four to six cell layers), characteristic of early preneoplastic alteration (Fig. 5e). It should be noted that in mice the normal urinary bladder mucosa contains no more than three layers of cells (26).
Fig. 5. Effect of halofuginone on progression of BBN-induced bladder carcinoma in mice. a, scheme describing the experimental design. b, 0.05% BBN in drinking water; ↓, halofuginone administration. b and c, macroscopic appearance of urinary bladders from untreated (left) and halofuginone-treated (right) mice. Bladder-specific carcinogen (BBN) was administered during the first 10 weeks (right and left). Halofuginone-containing diet was given starting on week 11 (right). Mice were sacrificed on week 26, and bladders were removed and photographed (×20) before (b) and after (c) embedding in paraffin, sectioning, and H&E staining. Note the well established full-thickness invasive bladder carcinoma induced by BBN in mice that did not receive halofuginone (c, left), as compared with the nearly normal appearance of bladder from halofuginone-treated mice (c, right). A similar appearance was obtained when the halofuginone therapy was initiated on weeks 1 or 6. d-f, microscopic appearance. BBN was administered in the drinking water during the first 10 weeks (d-f). Halofuginone-containing diet was given starting on week 11 (e) or 20 (f). Mice were sacrificed on weeks 26 (d and e) and 29 (f), and bladders were subjected to paraffin embedding, 5-μm thick sectioning, and H&E staining, ×200. d, carcinoma invading the full-thickness of the bladder is evident on week 26 in mice from control group (without halofuginone treatment). S, bladder serosa. e, only hyperplastic changes in the bladder mucosa are noted after halofuginone treatment initiated on week 11. N, region of normal, unchanged mucosa containing only three layers of urothelial cells. H, region of hyperplastic mucosa containing six to eight layers of cells. f, halofuginone treatment initiated on week 20. Only superficial bladder carcinoma with single cells invading the submucosa (arrows) was detected.
Table 1 Effect of halofuginone on BBN-induced bladder carcinogenesis: light microscopic evaluation of tissue sections

<table>
<thead>
<tr>
<th>Group</th>
<th>Halofuginone treatment (wks)</th>
<th>Normal mucosa</th>
<th>Preneoplastic changes</th>
<th>Superficial carcinoma</th>
<th>Invasive carcinoma</th>
</tr>
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<tbody>
<tr>
<td>1 (n = 8)</td>
<td>1–26</td>
<td>0</td>
<td>7</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2 (n = 6)</td>
<td>6–26</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>3 (n = 9)</td>
<td>11–26</td>
<td>1</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4 (n = 7)</td>
<td>20–29</td>
<td>0</td>
<td>4</td>
<td>3</td>
<td>0</td>
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<tr>
<td>5 (n = 9)</td>
<td>No treatment</td>
<td>0</td>
<td>2</td>
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* In two mice, single tumor cells invading the submucosa and part of the muscular layer was noted.

We have also tested the effect of halofuginone when administered beginning on week 20 after the initiation of BBN treatment when an established, often invasive tumor is already present in 80–100% of the mice, as demonstrated by us (26) and other investigators (25). Histological examination revealed that in about 50% of these mice (four of seven), only preneoplastic changes, primarily severe hyperplasia, were observed, whereas early carcinoma with superficial invasion was noted in the other three mice (Fig. 5). This is in contrast to mice that were not treated with halofuginone as described above, in which tumor invading the muscular layer and reaching the serosa was evident in seven of nine animals. These results clearly show that the initiation of halofuginone treatment even at an advanced stage of tumorigenesis halts further progression and may even lead to a partial regression of the invading tumor.

To further evaluate the effectiveness of halofuginone therapy in BBN-induced bladder cancer, we analyzed the levels of H19 gene expression in bladders derived from untreated and halofuginone-treated mice. Unlike normal adult tissues that never express the H19 gene, bladder cancer is a prototype of tumors that abundantly express H19 both in mice and in humans (26–29). In situ hybridization with H19-specific probe revealed a high level of H19 gene expression in both the epithelial and stromal compartments of bladder tumors induced by BBN (Fig. 6a). After halofuginone treatment, H19 expression was not detected in any tissue element of the bladder wall (Fig. 6b).

DISCUSSION

Our results demonstrate that halofuginone, a low molecular weight alkaloid compound known to inhibit collagen type α1(I) (18–21) and MMP-2 (23) gene expression, effectively suppresses the progression of primary tumors in both transplantable and chemically induced models of bladder cancer. A profound inhibition of MBT2-t50 primary tumor growth was observed in mice that received halofuginone on the day of tumor cell inoculation, as well as after the appearance of a detectable s.c. tumor. Similarly, halofuginone abrogated BBN-induced tumor in the mouse bladder and its progression toward a highly invasive carcinoma. Again, an antitumor effect of halofuginone was exerted even when the halofuginone therapy was initiated at advanced stages of tumorigenesis, such as hyperplasia, dysplasia, and even a well-established invasive cancer.

Several possible mechanisms for MBT2-t50 tumor growth suppression should be considered. In the present and in several previous studies, we reported that halofuginone exerts an antiproliferative effect on various cell types (21, 22). Nevertheless, the observed therapeutic effect of halofuginone cannot be attributed solely to its direct antiproliferative activity. Our data indicate that the role of halofuginone in tumor suppression is more complex than that of merely antiproliferative cytostatic agents. Rather, an effect on tumor angiogenesis and stromal support seems to play a decisive role. Plasma levels of halofuginone in mice that were maintained on halofuginone-containing diet were 2–3 ng/ml. It should be pointed out, however, that the actual concentration of halofuginone in tissues is considerably higher than in plasma, as indicated by a large apparent volume distribution of the drug (37, 38). It is, therefore, conceivable that the amounts of halofuginone in the tumor and stroma tissues reach the levels (20–100 ng/ml) in which the drug exerts its inhibitory effects on collagen α1(I) and MMP-2 gene expression and vascular tube formation in vitro.

We investigated the effect of halofuginone on neovascularization in the MBT2-t50 tumor model. A significant reduction in vascular density was revealed by histological examination of tumors derived from halofuginone-treated mice. This finding is supported by the observa-
tion that halofuginone efficiently inhibited vascular tube formation in collagen-embedded rat aortic rings. Our preliminary results indicate that p.o.-administered halofuginone also inhibits neovascularization in the mouse corneal micropocket assay. In view of the involvement of MMP-2 in tumor angiogenesis (12–17), suppression of the MMP-2 gene by halofuginone may play an important role in this inhibition. In a recent study, we have demonstrated a strong inhibitory effect of halofuginone on the MMP-2 promoter, leading to a marked decrease in MMP-2 enzymatic activity and matrix invasiveness of both bladder carcinoma (23) and vascular endothelial cells. The significance of MMP-2 in angiogenesis was recently emphasized by a marked inhibition of tumor induced angiogenesis and tumor progression in MMP-2-deficient mice (17). MMP-2 inhibitors are, therefore, regarded as promising candidates for cancer therapy (16, 17). In fact, some of these inhibitors are currently being tested in clinical studies (39, 40).

Apart from an effect on MMP-2, the antiangiogenic activity of halofuginone can be attributed to its efficient inhibition of collagen type α1(I) gene expression. A significant reduction in the abundance of collagen α1(I) transcripts, primarily in the stromal compartment of the MBT2 tumor, was demonstrated in the present study by in situ hybridization. It was proposed that type I collagen is involved in directing the migration and assembly of endothelial cells in newly formed blood vessels (41, 42). Exogenous type I collagen also promotes rapid tube formation by cultured confluent human dermal microvascular endothelial cells (43). The tubes contained collagen fibrils in the lumen spaces, suggesting that the endothelial cells use the fibrils to fold and align into tube structures (43). Moreover, transcriptional activation of the collagen α1(I) gene was observed in endothelial cells undergoing angiogenesis in vitro (42). Due to its inhibitory effect on both MMP-2 and collagen α1(I) gene expression, halofuginone seems to interfere with two critical steps in tumor vascularization, namely degradation of ECM by invading and migrating endothelial cells and their proper assembly into vascular tubes. The observation that halofuginone reduced the amount of collagen α1(I) in the stromal compartment of the MBT2-S0 tumor, as well as our previous studies demonstrating an inhibitory effect of halofuginone on ECM deposition (21, 22), suggest an additional mechanism for the antitumoral activity of halofuginone through a profound suppression of the tumor stromal support, known to promote tumor progression (11).

To evaluate the therapeutic effect of halofuginone under conditions that closely resemble tumorigenesis in human patients, we applied the BBN-induced bladder carcinoma mouse model. This model has been widely used to study the sequence of events occurring during bladder carcinogenesis (24–26). Chemical carcinogenesis simulates the major pathogenic mechanism known to be involved in human bladder carcinoma. In this model, a stepwise alteration in normal bladder epithelium, from simple to severe hyperplasia and dysplasia, followed by superficial carcinoma and, finally, invasive carcinoma, can be evaluated by histological examination of the bladder tissue (25, 26). We have initiated oral halofuginone treatment at different time periods corresponding to these stages of tumor progression. In seven of nine mice that were not treated with halofuginone, tumor invading the full-thickness of the bladder (i.e., sub-mucosa, muscular layer, serosa) was noted. In contrast, invasive cancer could not be detected in any of the 23 mice that were treated with halofuginone at the different stages of tumor progression mentioned above. Histological examination of bladder tissue derived from these mice revealed primarily hyperplastic alterations characteristic of an early preneoplastic stage. Administration of halofuginone at week 20, when an established tumor was already formed, prevented the progression into deep invasive carcinoma. Only superficial carcinoma (three of seven mice) or advanced preneoplastic changes (four of seven mice) were observed at week 29. It seems that halofuginone effectively prevented the transition of hyperplastic bladder epithelium into malignant invasive tumor. BBN-induced hyperplastic changes occurred to a similar extent regardless of whether halofuginone was administered at weeks 1, 6, or 11. This result indicates that halofuginone has little or no effect on the early proliferative stage of tumorigenesis, as compared with a marked inhibition of tumor establishment and invasiveness, consistent with an effect on the tumor stromal support and vascularization, as also noted in the MBT2 transplantable tumors. Clearly, tumor invasiveness, angiogenesis, and stromal support are critical for the transition from a preneoplastic stage into a highly malignant tumor.

Another indication of the anticancerous effect of halofuginone was the absence of H19 gene expression in halofuginone-treated mice as compared with its high level in BBN-induced bladder tumors in mice that did not received halofuginone. We have previously demonstrated that in this system the H19 gene is expressed initially in the stroma concomitant with mucosal hyperplasia, and later on in the developing tumor (26). H19 is an oncodevelopmental gene, tightly linked to tumorigenesis in different organs, particularly the bladder. The course of H19 expression in BBN-induced bladder cancer in mice is essentially the same as in human patients (26). As expected, H19 was highly expressed in BBN-induced bladder carcinoma. Treatment with halofuginone, which prevented the transformation of the hyperplastic bladder mucosa into neoplastic tissue, resulted in an almost complete absence of H19 gene expression both in the stromal and epithelial compartments. A number of reports relate H19 expression to blood vessel development (44, 45). Hence, lack of H19 transcripts in halofuginone-treated bladder could be related to the observed antiangiogenic effect of halofuginone.

Altogether, our results suggest that in both transplantable and chemically induced bladder tumors halofuginone exerts a profound anticancerous effect through a combined action on several critical determinants of tumor progression, such as angiogenesis, stromal support, and cell proliferation. Halofuginone is a p.o. available low molecular weight alkaloid compound that has long been approved for use as an antiparasitic agent in farm animals, mainly chickens (46). Its inhibitory effects on angiogenesis and tumor progression were reversible, and no toxic effects were observed in mice receiving halofuginone (70 μg/kg body weight/day) during 2 years (LD50 = 4 mg/kg). This, together with its oral availability, makes halofuginone a likely candidate for further evaluation in cancer treatment.

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