A Peptide with Three Hyaluron Binding Motifs Inhibits Tumor Growth and Induces Apoptosis

Xue-Ming Xu, Yixin Chen, Jinguo Chen, Shanmin Yang, Feng Gao, Charles B. Underhill, Karen Creswell, and Lurong Zhang

Department of Oncology, Lombardi Cancer Center, Georgetown University Medical School, Washington, DC 20057

Abstract

A number of hyaluronan (HA) binding proteins such as soluble CD44, receptor for hyaluronan-mediated motility (RHAMM), and metastatin inhibit tumor growth and metastasis. To determine whether the HA binding motif is the element responsible for the antitumor effect of this family of proteins, we examined the biological activity of a 42-amino acid peptide (designated as BH-P) that contains three HA binding motifs [B(X7)B] from human brain HA binding protein. In initial experiments with cultured cells, we found that synthetic BH-P inhibited the proliferation and colony formation of tumor cells. It also blocked the growth of tumors on the chorioallantoic membranes of 10-day chicken embryos. In addition, MDA-435 melanoma cells that had been transfected with an expression vector for BH-P grew at a slower rate in nude mice than the vector-alone transfectants. Final studies revealed that the BH-P could activate caspase-8, caspase-3, and poly(ADP-ribose) polymerase and trigger the apoptosis of tumor cells. Taken together, these results suggest that the HA binding motif that is present in HA binding proteins may be responsible for the antitumor effect exerted by the members of this family.

Introduction

The members of the family of HA binding proteins (HABPs) can be divided into two major groups: membrane-bound forms, such as soluble CD44 and receptor for HA-mediated motility (RHAMM), and extracellular forms, such as aggrecan (3) and link protein (4). These HABPs have been implicated in a number of cellular functions such as migration, adhesion (5), growth, differentiation, and apoptosis (6, 7, 8). Many of these functions are important in tumor progression (6, 9, 10).

In contrast to membrane-bound HA receptors, the truncated soluble forms of HA receptors have been found to possess antitumor activity (11–13). For example, a soluble recombinant CD44 disrupted the interaction between CD44 and HA and inhibited tumor formation and metastasis (11, 12). Similarly, a soluble form of RHAMM blocked the ability of tumor cells to form lung metastases (13). Importantly, clinical data have demonstrated that a high level of soluble CD44 in the serum was associated with a favorable clinical outcome in ovarian cancer (14). Conversely, low serum levels of soluble CD44 variant 6 were associated with poor prognosis of patients with pancreatic carcinoma (15). These findings suggest that soluble HA receptors shed from tumor cells might trigger a signaling pathway to block tumor cell growth.

In addition, the extracellular forms of HABPs also exhibit antitumor activity. The most notable example is cartilage, which contains particularly large amounts of HABPs (16). Because cartilage is an avascular tissue and relatively tumor resistant (17), thousands of cancer patients have taken oral preparations of cartilage as an alternative medicine, based on a belief that it contains natural antitumor substances. Although the therapeutic value of this approach is still controversial (18, 19), in some patients, cartilage does exhibit an anticancer activity (20–23). Indeed, several proteins and peptides isolated from cartilage have been found to have both antitumor and antiangiogenic activity (24–27). Along these lines, we have purified HABPs from bovine cartilage by affinity chromatography and found that it inhibits tumor growth and angiogenesis (28).

Because several different members of the HABP family have inhibitory effects on tumor growth, metastasis, and angiogenesis (28–29), it is possible that the commonly shared HA binding motif B(X7)B of HABPs is responsible for these effects. B(X7)B is the minimal amino acid composition required for HA binding and consists of two basic amino acids (either arginine or lysine) flanking a sequence of seven amino acids (29). It is highly conserved in members of the HABP family, and each member contains several such motifs. We speculate that the peptide that contains HA binding motifs might also exert antitumor activity.

To test this hypothesis, we synthesized or expressed BH-P containing 42 amino acids with three HA binding motifs derived from human brain HABP and examined its antitumor activity both in vitro and in vivo. The results suggest that the peptide enriched in HA binding motifs possesses an antitumor activity that may be mediated by the induction of apoptosis.

Materials and Methods

Synthesis of BH-P. BH-P (CNRRCGGRRAVLGSPRVKTFLSGRRGGRVQRKVXNEAYRF) contains three HA binding motifs from the NH2 terminus of the human brain HABP (GenBank accession number AY007241). It has a molecular mass of 4,736 and an isoelectric point of 12.01. The control peptide (control-P) consists of the same amino acids with a scrambled sequence. To impede enzymatic degradation, the NH2 terminus of the synthetic peptide was acetylated, and the COOH terminus was amidated. Before use, the peptides were dissolved in dimethylformamide and 1% acetic acid, diluted with saline to a concentration of 1 mg/ml, and sterilized by boiling for 15 min.

HA Binding Assay. Fifty μg of BH-P or control-P in 100 μl of PBS were mixed with 20 μl of [3H]HA (5 × 105 cpm/μg, 490 μg/μl; Ref. 30) in the presence and absence of 50-fold excess of cold HA, incubated for 1 h, and then applied to nitrocellulose membranes on a dot blot apparatus. The free [3H]HA...
was washed away with PBS, and each dot was cut out individually and counted for radioactivity.

**Cell Proliferation Assay.** Aliquots of complete medium containing 5,000 MDA-435 melanoma cells were distributed into a 96-well tissue culture plate. On the following day, the medium in each well was replaced with 160 µl of fresh medium and 40 µl of solution containing different concentrations of the peptides. One day later, 0.3 µCi of [3H]thymidine in 30 µl of serum-free medium was added to each well. After 8 h, the cells were harvested, and the amount of incorporated [3H]thymidine was determined with a beta counter. In some cases, 200 µg/ml of HA (Lifecore, Chaska, MN), 20 units/ml of testicular hyaluronidase (H4272; Sigma, St. Louis, MO), 40 µM Z-VDVAD-FMK (a pan-caspase inhibitor), or 20 µM Z-VAD-FMK (an inhibitor for caspase-2, -3, and -7) was added with the peptides to determine whether the effects of BH-P could be blocked.

** Colony Formation Assay.** Twenty thousand MDA-435 cells were suspended in 1 ml of 0.36% agarose in complete medium containing 100 µg/ml of control-P or BH-P, and then the cells were placed immediately on the top layer of BH-P solid agarose in complete medium in 6-well plates. Two weeks later, the number of colonies > 60 µm in diameter was determined using an Omnicon Image Analysis System (Imaging Products International, Inc., Chantilly, VA).

**Tumor Growth on the Chicken CAM.** Three million MDA-435 cells or one million B16 melanoma cells were placed on top of the CAMs of 10-day-old chicken embryos (15 eggs/group) and incubated at 37.8°C for 2 days to allow the tumors to become established. Then, 200 µg of control-P or BH-P in 200 µl of PBS were either i.v. injected into the CAMs once (in the B16 model) or trophically administered onto the tumor xenografts (in MDA-435 model) on the CAMs on a daily basis. Five days later, the xenografts were dissected from the membrane, photographed, and weighed.

**Construction of Expression Vector of BH-P and Transfection of MDA-435 Cells.** The BH-P amino acid sequence was back-translated into a cDNA sequence. (5'TGG ACG CGT TGC GGT CGT GTT CTGGTCTCCCGTGGTAAA TGG ACC TCC CTGGT CGGT CGT GTG CTG GGT CGT GTT CTG GGT AAA TGG ACG CGT TGC GGT CGT GTT CTG GGT AAA TGG ACG CGT TGC GGT CGT TGC GGT GGT CGT CGT GCT GTT

**Cell Proliferation**

**Cell Binding Assay.** FITC-BH-P (1 µg/ml), with or without 100-fold excess BH-P, was added to MDA-435 cells cultured on cover slips and incubated at 4°C for 30 min. The cells were washed with cold PBS and fixed with freshly prepared, buffered 4% formaldehyde and then examined with a confocal microscope.

**Cell Surface HA Binding Assay.** MDA-435 or TSU tumor cells were harvested with EDTA-PBS and pretreated with 50 units/ml of testicular hyaluronidase at 37°C for 30 min and then mixed with FITC-BH-P (1 µg/ml) with or without a 100-fold excess of HA at 4°C for 30 min. The binding of FITC-BH-P to the cell surface was analyzed by flow cytometry.

**FITC-Dextran Staining.** To determine whether BH-P damaged the plasma membrane, we probed the cells with FITC-dextran. MDA-435 cells at 80% confluence were treated with 100 µg/ml of control-P or BH-P overnight, harvested with 5 mM EDTA in PBS, washed, and resuspended in 10% calf serum, 90% DMEM and incubated for 1 h to allow the cells to recover from possible damage during harvesting. The cells were then treated with 5 µg/ml of FITC-dextran (Molecular Probes, Eugene, OR) for 10 min at room temperature, fixed with 4% paraformaldehyde, and subjected to flow cytometry analysis.

**Western Blotting.** Both cultured MDA-435 cells and tumor xenografts formed by MDA-435 transfected cells were analyzed for apoptosis-related molecules. Cultures of MDA-435 cells at ~80% confluence were treated with 100 µg/ml of either control-P or BH-P overnight and then harvested with 1.0 ml of lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.5 mg/ml leupeptin, 1 mM EDTA, 1 mg/ml phosphatase, and 0.2 mM phenylmethylsulfonyl fluoride). The tumors (25–30 mg) formed by MDA-435 transfecteds in nude mice were immersed in liquid nitrogen, homogenized, and lysed in 1.0 ml of lysis buffer. The concentration of protein in cell lysate was determined with the BCA reagent (Pierce, Rockford, IL), and 20 µg of protein from each lysate were loaded onto a 4–12% BT NuPAGE gel, electrophoresed, and transferred to a nitrocellulose membrane. The membranes were blocked with 5% nonfat milk in PBS for 30 min and then incubated for 1 h with 1 µg/ml primary antibodies against caspase-2, caspase-3, PARP, and α-tubulin and followed by peroxidase-labeled secondary antibodies and Super-signal Western reagents (Pierce).

**Detection of DNA Fragmentation.** MDA-435 cells and TSU human bladder cancer cells were cultured with either 100 µg/ml control-P or BH-P for 24 h, harvested with 10 mM EDTA, and washed once with PBS. The cell pellets were suspended in 1 ml of buffer (150 mM NaCl, 50 mM EDTA, pH 8.0, 1% SDS, and 0.5 mg/ml of proteinase K) and incubated at 55°C for 16 h. After extraction with phenol and chloroform, the genomic DNA was precipitated with ethanol. The DNA pellets were dissolved in 100 µl of TE buffer [10 mM Tris (pH 8)-1 mM EDTA], and then 15 µl of samples were electrophoresed in a 1.5% agarose gel, stained with ethidium bromide, and photographed.

**Statistical Analysis.** The mean and SE were calculated from the raw data and then subjected to Student’s t test. P < 0.05 was regarded as statistically significant.

**Results.**

**Synthetic BH-P Binds to HA.** In initial experiments, we tested the synthetic BH-P for its ability to bind to HA. As shown in Fig. 1A, BH-P had significant [3H]HA binding activity that could be blocked by a 50-fold excess of cold HA. In contrast, the control-P showed little or no binding to [3H]HA, indicating that the binding of BH-P to HA was specific.

**BH-P Inhibits the Growth of Tumor Cells in Vitro.** When BH-P was added to the medium of cultured MDA-435 cells for 18–24 h, it caused the cells to become rounded and detached, whereas the control peptide had no such effect (data not shown). To quantitatively measure the extent of this inhibitory effect, the cells were subjected to a [3H]thymidine incorporation assay. Figure 1B shows that the proliferation of MDA-435 tumor cells was inhibited by BH-P in a dose-dependent manner with an EC50 of ~100 µg/ml. In contrast, control-P had no effect, even at a concentration of 200 µg/ml (Fig. 1B, first column). In addition, BH-P also inhibited the colony formation of these tumor cells under anchorage-independent conditions on soft agar (Fig. 1C).

When BH-P was tested on a panel of cultured cells, its effects varied depending on the cell type. BH-P appeared to have a greater effect on tumor cells than on the relatively normal cell lines Cos-7 (green monkey kidney cells) and NIH-3T3 (fibroblast cells; data not shown).

**BH-P Inhibits Tumor Growth in Vivo.** In view of these in vitro results, we then examined the effects of BH-P in vivo in two different model systems.

In the first model system, BH-P was directly injected into a vein of chicken embryo CAMs on which tumor xenografts were growing. As
shown in Fig. 2A, the sizes of tumor xenografts formed by B16 melanoma cells that had been i.v. injected with BH-P were much smaller than those injected with control-P. Similar results were obtained with MDA-435 cells in the same model (Fig. 2B). Furthermore, the difference in the tumor weights between the test and control groups was statistically significant (Fig. 2, C and D). The fact that BH-P demonstrated an inhibitory effect on the growth of both B16 melanoma and MDA-435 cells suggested that the antitumor effect of BH-P was not limited to one cell line and was likely to be universal.

The second model system consisted of gene transfection, which has the following advantages: (a) it results in a high concentration of BH-P in the tumors, which could not be achieved by systemic administration of synthetic BH-P; (b) BH-P should be evenly distributed in the tumors; and (c) a consistent level of BH-P could be maintained in

![Fig. 1. Binding of BH-P to HA and the inhibitory effects of BH-P on the growth of tumor cells.](image)

A, binding of BH-P to HA. Fifty μg of BH-P or control-P in 100 μl of PBS were mixed with 20 μl of [3H]HA without or with a 50-fold excess of cold HA for 1 h and then were transferred to a nitrocellulose membrane on a dot blot apparatus. After washing, the complex of [3H]HA and BH-P that had bound to the filter was counted for radioactivity. BH-P binds to [3H]HA, which could be abolished by an excess of HA. B, BH-P inhibited the proliferation of MDA-435 tumor cells in a dose-dependent manner. TdR, thymidine. C, BH-P reduced the ability of MDA-435 cells to form colonies on soft agar. The results represent the mean (n = 3) of a representative experiment, bars, SE. Each experiment was repeated at least three times with similar results. *, P < 0.05.

![Fig. 2. Inhibition of tumor growth on CAM.](image)

Two days after B16 melanoma cells (1 × 10^6) or MDA-435 cells (3 × 10^6) were placed on top of the CAMs of 10-day-old chicken embryos, 200 μg of control-P or BH-P in 200 μl of PBS were either i.v. injected into the CAMs once or tropically administrated onto the tumor xenografts on a daily basis. Five days later, the tumors were harvested, photographed (A and B), and weighed (C and D). Compared with the control-P, the tumors treated with BH-P were smaller (P < 0.05). Bars, SE.

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the tumors. For this approach, the cDNA sequence coding for BH-P was obtained by back-translation and then inserted into a pSecTag vector that had a cytomegalovirus promoter and an IgG signal peptide for secretion. The MDA-435 cells were transfected with either a pSecTag empty vector or a BH-P-pSecTag expression vector. To avoid clonal variation, the transfectants that survived in G418 selection medium and expressed BH-P were first pooled and then injected into the mammary fat pads of nude mice. Fig. 3A shows that the growth curve of the MDA-435 cells transfected with BH-P was below that of the mock-transfected cells. Forty-two days after inoculation, the average size of tumor xenografts in the BH-P group was smaller than that in the control group (Fig. 3B), and the difference in tumor weight between the two groups was statistically significant (Fig. 3C; P < 0.05).

Thus, the results from two different experimental models were consistent with each other, strongly indicating that BH-P could inhibit tumor growth in vivo. Importantly, when BH-P was administered at a concentration that inhibited tumor growth, there was no notable side effect on either the chicken embryos or mice with regard to animal body weight and activity assessed at the end of each experiment (data not shown).

**BH-P Binds to the Plasma Membrane and Causes an Increased Permeability.** To determine whether there was a physical association of BH-P with the targeted cells, a binding assay was preformed. MDA-435 cells were incubated with FITC-BH-P at 4°C for 30 min and examined under a confocal microscope. Green fluorescence clearly outlined the tumor cells, suggesting that FITC-BH-P bound to the plasma membranes (Fig. 4A). This could be blocked by a 100-fold excess of BH-P, indicating that the BH-P specifically bound to the cell surface.

Presumably, BH-P was binding to HA or other glycosaminoglycans on the cell surface. To test for this, we preincubated the tumor cells with testicular hyaluronidase to digest HA and chondroitin sulfate on the plasma membranes (Fig. 4B). This could be blocked by a 100-fold excess of BH-P, indicating that BH-P specifically bound to the cell surface.

Next, we wanted to determine whether BH-P damaged the plasma membrane. To address this issue, we used high molecular weight FITC-dextran (40,000), which is normally excluded from the cytoplasm of cells with intact plasma membranes. However, when MDA-435 cells were treated with 100 μg/ml of BH-P, the percentage of cells that stained positively for FITC-dextran increased from 12% in control-P-treated cells to 37% in those treated with BH-P (Fig. 4C), indicating that the permeability of the plasma membrane was increased with the BH-P treatment.

**BH-P Triggers Apoptosis.** To determine whether BH-P triggered apoptosis, both MDA-435 and TSU tumors cells were treated with BH-P or control-P, and then the DNA was extracted and electrophoresed on a 1.5% agarose gel. Fig. 4D shows DNA ladderizing in BH-P-treated tumor cells but not in the control-P-treated cells, suggesting that BH-P could trigger apoptosis.

We then examined the activation of molecules that are part of the apoptotic pathway by Western blotting. Fig. 4E shows that caspase-8, caspase-3, and PARP were activated in both tumors formed by cells transfected with BH-P expression vectors in nude mice and in cultured tumor cells as compared with their control counterparts. To determine whether blocking apoptosis could prevent the inhibitory effects of BH-P on tumor cells, Z-VAD-FMK (40 μM; a pan-caspase inhibitor) and Z-VDVAD-FMK (20 μM; an inhibitor for caspase-2, -3, and -7) were added to tumor cells treated with (as test) or without (as control) BH-P. The result demonstrated that although the caspase inhibitors alone did not have any effect on the cell proliferation, the inhibitory effect of BH-P on tumor cells could be blocked by the addition of Z-VAD-FMK or Z-VDVAD-FMK (data not shown), which strongly suggests that the BH-P inhibition of tumor growth is associated with caspase-related apoptosis.

**Discussion**

The results of this study demonstrate that BH-P, a peptide enriched with (X₄₀)B motifs that bind to HA, can exert antitumor effects as indicated by the following: (a) BH-P inhibits the proliferation of tumor cells growing under both anchorage-dependent and-independent conditions; and (b) BH-P inhibits tumor growth in vivo in both the chicken embryo CAM and nude mice xenograft models. This inhibitory effect of BH-P was tested with MDA-435 human melanoma cancer cells, B16 melanoma cells, and TSU human bladder cancer cells. In each case, BH-P exerted a similar inhibitory effect, suggesting that the effect of BH-P is not cell line specific but is a universal phenomenon. Notably, there were no obvious side effects on either the development of the chicken embryos or on the body weights of the
mice, suggesting that BH-P spares normal cells and preferentially blocks the growth of malignant tumor cells. BH-P appears to exert its antitumor activity through its ability to induce apoptosis. The first step in this process is the binding of BH-P to the cell surface, which was suggested by confocal analysis with FITC-BH-P (Fig. 4A). This binding presumably involves HA or its related molecules (chondroitin sulfate), as indicated by flow cytometry analysis in which the treatment with excess HA or hyaluronidase blocked the binding of FITC-BH-P to the cell surface (Fig. 4B). Importantly, the interference of BH-P binding to cell surface HA reversed its effects on cell proliferation, suggesting that HA binding on the cell surface is necessary for the effect of BH-P. After binding and internalization, BH-P appears to trigger the apoptosis cascade, as indicated by the activation of critical molecules in the apoptosis pathway, such as caspase-8, caspase-3 and PARP, eventually resulting in the fragmentation of DNA. Indeed, the fact that several caspase inhibitors reversed the inhibitory effects of BH-P on cell proliferation strongly suggested that its effect was related to the induction of apoptosis. The increased permeability of the plasma membrane upon the treatment with BH-P could result from either direct damage caused by the binding of BH-P to the cell surface or as a consequence of intracellular apoptosis on the membrane. At present, the initial target of BH-P is unclear.

Although this is the first report regarding a peptide enriched in HA binding motifs having an antitumor activity, other biological effects have been attributed to HA binding peptides. For example, Mummert et al. (31) have reported that a 12-mer HA binding peptide, obtained from a screen of a peptide library with a phage display technique, could block HA-mediated leukocyte trafficking and inhibit inflammatory reactions. This 12-mer peptide has only one HA binding motif. In separate experiments, we found that a single HA binding motif is not sufficient to induce an antitumor effect (data not shown). According to our results, three HA binding motifs appear to be required to impart antitumor activity. We believe that the numbers of HA binding motifs in the peptides may determine their function. To block the HA-CD44 interaction that mediates the leukocyte migration, one HA binding motif seems sufficient (31). However, to trigger apoptosis in tumor cells, it seems to require a peptide that has more than one B(X7)B motif to simultaneously act on more than one site or more than one molecule on the cell surface.

Fig. 4. Effect of BH-P on apoptosis-related molecules. A, confocal microscopy shows the binding of FITC-BH-P to the surface of MDA-435 cells (top), which could be blocked by excess of BH-P (bottom). B, flow cytometry shows that the binding of FITC-BH-P to the cell surface was blocked by an excess of HA or preincubation with hyaluronidase. C, flow cytometry shows that treatment with BH-P resulted in an increased permeability of FITC-dextran, suggesting that the plasma membrane was damaged. D, analysis of DNA from MDA-435 and TSU cells indicates that treatment with BH-P results in laddering. E, Western blot analysis indicated that caspase-8, caspase-3, and PARP were activated in both tumors formed by BH-P transfectants and in cultured tumor cells treated with BH-P.
Recently, it has been reported that the binding of HA to CD44 could block apoptosis induced by antibodies to Fas (32). Directly, CD44 could physiologically trigger the up-regulation of Fas (33), and the activated T cells could use CD44 to undergo apoptosis (34). To weaken apoptosis, the Fas-triggering cells appeared to shed CD44 (35). These studies suggest that the HABP in conjunction with Fas weaken apoptosis, the Fas-triggering cells appeared to shed CD44 could physiologically trigger the up-regulation of Fas (33), and the block apoptosis induced by antibodies to Fas (32). Directly, CD44 helps to promote its antitumor activity; and (c) it should be easy to identify similar compounds to mimic antitumor activity of HABPs and peptides. The interruption of physiological function of native HABPs could result in an impairment in cell migration, invasion, and activation of apoptosis. In conclusion, basic research on the function of HA binding peptides may lead to the identification of other novel agents to trigger apoptosis and block tumor progression.

References
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