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To test our hypothesis that that the HA binding peptide may be a new anti-neurofibromatosis agent via inducing apoptosis, we have proposed to focus on three aims: **1)** To examine the anti-tumor effect of synthetic HA binding peptide on malignant neurofibromatosis cells; **2)** To examine the anti-tumor effect of genetically expressed targeted HA binding peptide; **3)** To examine the effect of targeted HA binding peptide on molecules involved in apoptosis.

The results of **first year** study indicated that: **1)** large scale synthesized HA binding peptide did possess HA binding activity; **2)** synthetic HA binding peptide exerted an anti-tumor effect of on ST88-14 NF1 cells; **3)** HA binding peptide could bind to Bcl-2/Bcl-x_L, which may be one of the mechanisms by which HA binding peptide inhibits ST88-14 NF1 cells; and **4)** the cells transfected with expression vector carrying cDNA of HABP could express this peptide.

The results of **this year** study demonstrated that: **1)** HA binding peptide is capable of reducing the level of phosphorylated ERK1; **2)** HABP reduces the level of cell cycle related molecules, such as cyclin B1 and cdc 2; **3)** HABP binds to Bcl-2 *in vivo* and induces apoptosis; and **4)** the effort has been made to set up the model system of NF1 tumor exnograft in mice for test the effect of HABP *in vivo*. **In the next year**, we will continue to examine the action mechanism of HABP and to test if the *in vitro* anti-tumor effect of HA binding peptide can be translated *in vivo* against the cell growth of neurofibromatosis.

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INTRODUCTION

The loss of NF1 gene is a major genetic characteristic of neurofibromatosis Type 1 (1-3). NF1 gene encodes a large cytoplasmic protein called neurofibromin (1). Similar to p53, neurofibromin is regarded as a tumor suppressor, since it plays a major role in the negative regulation of Ras activity which is crucial in cell survival signal transduction pathway. The loss of neurofibromin leads to the accumulation of hyperactive Ras-GTP due to a reduced conversion of active Ras-GTP to inactive Ras-GDP, which turns on uncontrolled mitogenic signals in the nucleus (2, 4-7). Therefore, NF1 can be regarded as a disease resulting from the disruption of the balance between cell proliferation and apoptosis (8, 9). In other words, NF1 cells gain immortality due to their over-proliferation and defective apoptosis.

Based on the molecular mechanism of NF1, several strategies can be utilized for the control of NF1.

First, it is highly likely that the inhibition of Ras-Raf-ERK/MAPK pathway can significantly reduce the growth of neurofibromatosis (10).

Secondly, the regulation of apoptosis can be used for the control of cell growth and the treatment of neurofibromatosis. For example, the down-regulation of anti-apoptotic molecule, such as bcl2, could reduce the growth of cells (11, 12).

Thirdly, the reduction of key molecules in the cell cycle is expected to suppress the tumor progression (13, 14).

We are interested in exploring the effect of hyaluronan (HA) binding proteins (HABPs) on the induction of apoptosis and the inhibition of the growth of tumor. This was based upon the following facts. **1)** Proteins that can bind to HA such as the soluble forms of CD44 and RHAMM can inhibit tumor growth and/or metastasis (15-17). **2)** Fragments of proteins that contain HA binding domain, such as endostatin (fragment of collagen XVIII), angiostatin (fragment of plasminogen), and hemopexin-like domain of metalloproteinase also possess potent anti-tumor activity (18-21). **3)** For more than a decade, the powder or extracts from shark cartilage have been widely used as alternative medicine by cancer patients in USA, Europe and Asia. In some patients, this substance did exhibit anti-tumor effects. Cartilage contains large amounts of HA binding proteins (HABP). It is possible that the anti-tumor effect of shark cartilage achieved in some patients is due to a small amount of HABP passing through impaired intestinal mucosa of these individuals (22-30). And **4)** several proteins purified from the cartilage, a HA-rich tissue, have been found to have the anti-tumor effect (31-36).

In this study, we propose to test our hypothesis that HA binding peptide may be a new anti-neurofibromatosis agent via inducing apoptosis. For this, we are focusing on three aims: **1):** To examine the anti-tumor effect of synthetic targeted HA binding peptide on malignant neurofibromatosis cells; **2):** To examine the anti-tumor effect of genetically expressed targeted HA binding peptide; and **3)**: To examine the effect of targeted HA binding peptide on molecules involved in apoptosis.

In past, we have finished the following tasks: **1)** chemical synthesis HA binding peptide and control peptide in a large scale; **2)** identification of its HA binding activity; **3)** characterization of anti-tumor activity of HA binding peptide; **4)** study of the effect of HA binding peptide on molecules involved in cell programmed death; and **5)** construction of mammalian expression vector for HA binding peptide.

We have demonstrated that: **1)** large scale synthesized HA binding peptide did possess HA binding activity; **2)** synthetic HA binding peptide exerted an anti-tumor effect of on ST88-14 NF1 cells; **3)** HA binding peptide could bind to Bcl-2/Bcl-x_L, the critical anti-apoptosis factors, which may be one of the mechanisms by which HA binding peptide inhibits ST88-14 NF1 cells; **4)** the cells transfected with expression vector carrying cDNA of HA binding peptide could express this peptide as evidenced by Western blotting.

In last year, we further determined the effect of HA binding peptide on the molecules related with cell cycle and apoptosis and the possible *in vivo* model for the administration of HA binding peptide. The data are summarized as follows.

BODY

It has been well demonstrated that the molecular basis for neurofibromatosis is the loss or mutation of NF1 gene, which leads to accumulation of hyperactive Ras-GTP, resulting in a constitutive mitogenic signaling of cell growth.

Based on our preliminary data and other published studies, we postulate that the targeted HA binding peptide may be a new anti-neurofibromatosis agent via inducing apoptosis. To test our hypothesis, we have proposed to focus on three aims: **1)** To examine the anti-tumor effect of synthetic targeted HA binding peptide on malignant neurofibromatosis cells; **2)** To examine the anti-tumor effect of genetically expressed targeted HA binding peptide; **3)** To examine the effect of targeted HA binding peptide on molecules involved in apoptosis.

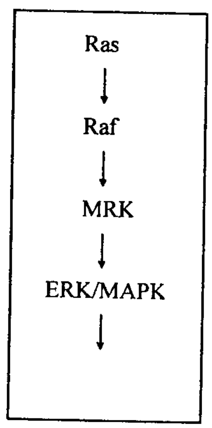
In the past year, we have performed the experiments: **1)** To determine if HA binding peptide (HABP) is able to inhibit the constitutive activation of Ras-Raf-ERK/MAPK pathway; **2)** To determine if HABP can interfere the cell cycle related molecules; **3)** To further study if HABP could induce apoptosis *in vivo* system; and **4)** To set up possible NF1 tumor model in mice.

The results are summarized as following:

1. HA binding peptide reduces the level of phosphorylated ERK1.

Since the loss of neurofibromin leads to the accumulation of hyperactive Ras-GTP due to a reduced conversion of active Ras-GTP to inactive Ras-GDP, the accumulation of hyperactive Ras-GTP results in a constitutive mitogenic signaling of cell growth (2, 4-7).

In the process of transduction the Ras signaling, the following pathway has been well identified:



It is obvious that signal through the RAS- ERK /MAPK pathway, phosphorylated ERK can be used as an indicator of when and where signaling is active.

Base on this fact of molecular error in NF1, we would like to see if the HABP can inhibit the ERK, which is the down-steam kinase responsible for further amplification of the hyperactive Ras-GTP function in NF1. For this, the ST88-14 cells, a typical line of NF1 cells, were plated in 100 mm dishes and treated with 100 μg/ml of control peptide or HABP (synthesized by experts of organic chemistry in Genemed Inc.). The cells were incubated with peptides for 24 hours and the cells were washed and harvested in lysis buffer (10 mM potassium phosphate at pH 7.5, 1 mM EDTA, 5 mM EGTA, 50 mM β-glycerophosphate, 1 mM sodium vanadate, 0.5% Triton X-100, 0.1% sodium deoxycholate, 1 mM magnesium chloride and 2 mM DTT). The protein concentration was determined with BCA method (PIERCE Inc). Thirty μg of lysate protein was load in 10% SDS-PAGE for electrophoresis. After transferring to the nitrocellulose membrane and blocking with 3% BSA-PBS, the total ERK protein and phosphorylated ERK (the functional form of ERK) were detected with anti-ERK or anti-phosphorylated ERK, respectively. The result (**Fig 1**) showed that while the total ERK was not affected by the treatment of ST88-14 cells with HABP, the phosphorylated ERK was reduced.

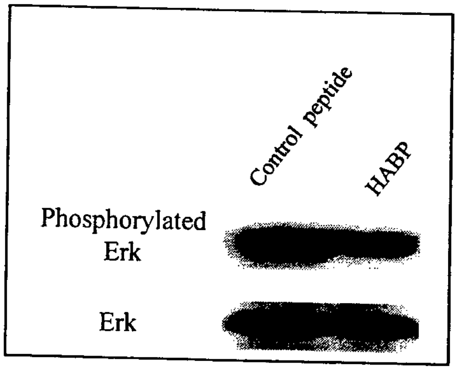


Fig 1. Blocking of phosphorylation of ERK with HABP. The ST88-14 cells were plated in 100 mm dishes and treated with 100 μg/ml of control peptide or HABP for 24 hours. The cell lysate was subjected to Western blot analysis. The result showed that while the total ERK was not affected by the treatment of ST88-14 cells with HABP, the phosphorylated ERK was reduced.

This data suggests that the HABP is capable of blocking the down-steam signaling of hyperactive Ras-GTP, the molecular error in NF1.

2. HA binding peptide reduces the level of cell cycle related molecules

The uncontrolled growth is one of the characteristics of NF1, which reflects a active process of cell cycle. The cyclins and their kinases (cdc) are the actual molecules that control the cell cycle. To determine the effect of HABP on the cyclins and their cdc, the Western blotting analysis was performed after the cells were exposed to HABP for 24 hours. The results (**Fig 2**) showed that while cyclin D1 was unchanged, the cyclin B1 and cdc2 were greatly reduced at the protein level.

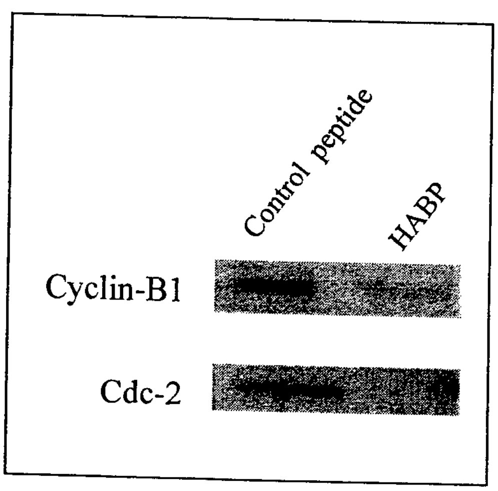


Fig 2. HABP Inhibition of cyclin B1 and cdc2. The tumor cells were treated with 100 μg/ml of control peptide or HABP for 24 hours. The cell lysate was subjected to Western blot analysis. The result showed that the levels of cyclin B1 and cdc2 were greatly reduced.

The above data indicates that the HABP interacts with functional proteins at both the RAS-ERK /MAPK pathway and the cell cycle related cyclin B1 and cdc2. This multi-levels control is favorable for HABP as a potential anti-NF1 agent.

3. HABP binds to Bcl-2 in vivo and induces apoptosis.

It has been proved that Bcl-2/Bcl-x_L, the critical anti-apoptotic molecules, are anchored on membranes and may form a large macromolecular structure or lattice, which stabilizes the membrane of mitochondria and prevents the cells from apoptosis. The functional blockade of Bcl-2 / Bcl-x_L could restore the apoptotic process, and thereby, could inhibit the uncontrolled proliferation of NF1 cells.

In the first year, we were exciting about out findings that: **1)** HABP could bind to Bcl-2/Bcl-x_L as assayed with ELISA like system; and **2)** HABP could interact with recombinant Bcl-2 *in vitro*.

We then wanted to see if this interaction could occur *in vivo*. To determine this, the tumor cells cultured in 100 mm dishes were first transfected with mammalian expression vector containing cDNA cording for Bcl-2 with GFP tag for 24 hours, and then incubated with biotinylated HABP or biotinylated control peptide for 3 hours and harvested in 1.5 ml of lysis buffer (150 mM NaCl, 1 mM EDTA, 20 mM. Tris-HCl pH 8.0, 0.5% Nonidet P-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1mM PMSF). One ml of cell lysates were mixed with strepavidin-sepharose beads and incubated overnight at 4°C. The beads were washed to get rid of miscellaneous protein, and then eluted with 30 μl of SDS loading buffer and boiling. Ten μl of transfected cell lysate was used as control. The samples were analyzed in Western blotting using anti-Bcl-2 antibody to detect the complex of HABP-Bcl-2-GFP.

The result (**Fig 3**) showed that the HABP could bind to Bcl-2 *in vivo* system.

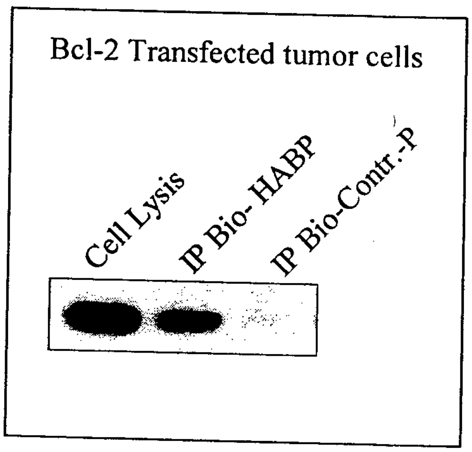


Fig 3. HABP binds to Bcl-2 *in vivo* system. The tumor cells cultured in 100 mm dishes were first transfected with mammalian expression vector containing cDNA cording for Bcl-2 with GFP tag for 24 hours, and then incubated with biotinylated HABP or biotinylated control peptide. The cell lysates were mixed with strepavidin-sepharose beads. The bound HABP-Bcl-2-GFP complex was detected using Western blotting with anti- Bcl-2 antibody.

Then, we wanted to examine if the interaction of HABP with Bcl-2 resulted in an apoptosis, since the critical anti-apoptotic molecule was disrupted. For this, tumor cells were treated with HABP or control peptide at a dose of 100 μg/ml. Twenty-four hours later, the DNA was harvested and subjected to the DNA ladder analysis.

The result (**Fig 4**) demonstrated that HABP indeed induced the apoptosis as determined by the DNA ladder analysis, a golden standard for the apoptosis.

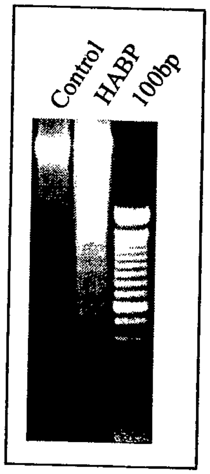


Fig 4. HABP induces apoptosis. The tumor cells were treated with HABP or control peptide at a dose of 100 μg/ml for 24 hour and then the DNA was harvested and subjected to the DNA ladder analysis.

What is the underlying mechanism of this phenomenon? Why the binding of HABP could induce apoptosis. We speculate that HABP acts in a fashion similar to pro-apoptotic molecule in Bcl-2 family, such as such as Bim, Bid, Bak, Bad, Bax, Bcl-xs, Blk, Bnip3, Bik and Hrk (37-39). To see if this is the case, we searched the existing database for the similarity between the HABP and pro-apoptotic molecules.

The HABP consists of 17 residues of amino acid: **KWCFRVCYRGICYRRCR,** which contains two typical $BX_{6-7}B$ motifs, which is six to seven neutral amino acids franked by two basic amino acids (R: arginine; K: lysine; H: histine). Interestingly, this $BX_{6-7}B$ motif exists in all the pro-apoptotic Bcl-2 family proteins examined **(Table 1)**. Some of them have more than one $BX_{6-7}B$ motif. Whether this similarity confers the HABP with the ability to bind to Bcl-2 will be further investigated.

Table 1. The similarity of HABP to pro-apoptosis proteins in BCL-2 Family ($BX_{6-7}B$ motif)

HABP:	KWCFRVCYRGICYRRCR		
Bim_L	HPRMVILRLLRYIVRLVWRMH		
Bim_S	RFIFRLVWRRH		
Bid	RSSHSRLGR	RTYVRSLAR	KKVASHTPSLLR
Bak	RLAL HVYQH	HHCIARW IAQR	
Bad	KKGLPRPK	RYGRELRR	RQSSSWTR
Bax	KLVLKALCTK	KKLSECLKR	
Bcl-xS	RKGQERFNR	HSSSLDAR	
Blk	KNNMKVAIKTLK	RQLLAPINK	
	RQSLRLVRK	KGAFSLSVK	RWFFRSQGRK
Bnip3	KHPKRTATLSMRNTSVMKK		
Bik	KENIMRFWR		
Hrk	HQRTMWRRRARSRR		

The motif or structure similarity of HABP with pro-apoptotic proteins in Bcl-2 may lay down the foundation for HABP being a novel death regulator for NF1.

4) Effort to set up the NF1 tumor model in mice.

To test the anti-tumor activity of HABP *in vivo*, the best model system is to use mouse model. Before we test the effect, we wanted to examine the growth behavior of the untreated NF1 cells in mice.

In vitro, the ST88-14 cells grow faster than other two cell lines (NF90-8 and NF88-3) derived from patients of neurofibromatosis. However, when we subcutaneously injected 2.5 x 10^6 ST88-14 cells into nude mice, we did not observe the growth of tumor. We speculated that this might due to insufficiency of cells injected. Then, we injected 10^7 ST88-14 cells, but the tumor was not formed.

To increase the possibility of forming tumor exnograft in mouse, we decided to try SCID (severe combined immunodeficient) mice that defect in both T lymphocytes and B lymphocytes. Compared with the nude mice that defect only T lymphocytes, the SCID mice confer a less capability of rejection of the implanted tumor cells. However, to our dismay, the 10^7 ST88-14 cells still could not form tumor in SCID mice.

We then tired to implant the NF1 cells in organs that have a less extent of immune surveillance and more extent of the supply of nutrition, such as liver. Five million of ST88-14 cells were injected into sub-capsule of liver. Still, to our disappointment, the tumor nodule did not form.

Since the growth of NF1 tumor in mice is prerequisite for test the effect of HABP *in vivo*, we are actively seeking for the advice from experts in this field. We are also looking for the new NF1 cell lines that might have better potential to form tumor exnograft in mice.

Once the cell line and the *in vivo* model system are defined, we will use the synthetic HABP and its expression vector that we have been obtained in the past to test the *in vivo* effect of HABP on NF1.

In summary, in the past year, we have demonstrated that: **1)** HA binding peptide is capable of reducing the level of phosphorylated ERK1; **2)** HABP reduces the level of cell cycle related molecules, such as cyclin B1 and cdc 2; **3)** HABP binds to Bcl-2 *in vivo* and induces apoptosis; and **4)** the effort has been made to set up the model system of NF1 tumor exnograft in mice for test the effect of HABP *in vivo*.

In the next year, we will continue to examine the action mechanism of HABP and to test if the *in vitro* anti-tumor effect of HA binding peptide can be translated *in vivo* against the cell growth of neurofibromatosis.

Key Research Accomplishments

In past second year, we have demonstrated that: **1)** HA binding peptide is capable of reducing the level of phosphorylated ERK1; **2)** HABP reduces the level of cell cycle related molecules, such as cyclin B1 and cdc 2; **3)** HABP binds to Bcl-2 *in vivo* and induces apoptosis; and **4)** the effort has been made to set up the model system of NF1 tumor exnograft in mice for test the effect of HABP *in vivo*.

Conclusions

- HA binding peptide reduce the ERK1 phosphorylation, which is the down-steam key kinase for conducting the function of hyperactive Ras-GTP, due to the loss or mutation of NF1 gene.

- HABP reduces the level of cell cycle related molecules, such as cyclin B1 and cdc 2, which sets up another breaker to stop the over-proliferation of NF1 cells.

- HABP binds to Bcl-2 *in vivo* and induces apoptosis, which may due to its molecular structure similarity to the pro-apoptotic molecule in Bcl-2 family, such as such as Bim, Bid, Bak, Bad, Bax, Bcl-xs, Blk, Bnip3, Bik and Hrk.

Reportable outcomes

(Due to or partially due to this support)

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1. Yang S, Chen J, Guo Z , Xu X, Wang L, Pei X, Yang Y, Underhill CB and Zhang L: Triptolide Inhibits the Growth and Metastasis of Solid Tumors. Mol Cancer Ther. 2003; 2(1):65-72.

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