Tachyplesin Activates the Classic Complement Pathway to Kill Tumor Cells

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Abstract

Tachyplesin is a small, cationic peptide that possesses antitumor properties. However, little is known about its action mechanism. We used phage display to identify a protein that interacted with tachyplesin and isolated a sequence corresponding to the collagen-like domain of C1q, a key component in the complement pathway. Their interaction was subsequently confirmed by both ELISA and affinity precipitation. Tachyplesin seemed to activate the classic complement cascade because it triggered several downstream events, including the cleavage and deposition of C4 and C3 and the formation of C5b-9. When TSU tumor cells were treated with tachyplesin in the presence of serum, activated C4b and C3b could be detected on tumor cells by flow cytometry, Western blotting, and confocal microscopy. However, this effect was blocked when the tumor cells were treated with hyaluronidase or a large excess of hyaluronan, indicating that hyaluronan or related glycosaminoglycans were involved in this process. Treatment of cells with tachyplesin and serum increased in membrane permeability as indicated by the ability of FITC-dextran to enter the cytoplasm. Finally, the combination of tachyplesin and human serum markedly inhibited the proliferation and caused death of TSU cells, and these effects were attenuated if the serum was heat-inactivated or if hyaluronidase was added. Taken together, these observations suggest that tachyplesin binds to both hyaluronan on the cell surface and C1q in the serum and activates the classic complement cascade, which damages the integrity of the membranes of the tumor cells resulting in their death. (Cancer Res 2005; 65(11): 4614-22)

Introduction

Small peptides represent a promising new group of compounds in the fight against cancer (1–3). Of particular interests are naturally occurring peptides that have antimicrobial activity, many of which also have antitumor activity (4–6). To date, >100 antibiotic peptides have been described and these can be arbitrarily classified into three major groups based on their structure: linear peptides, disulfide-linked peptides (such as defensins and tachyplesins; refs. 7–9), and those containing posttranslationally modified amino acids. In most cases, these peptides contain cationic amino acids that can interact electrostatically with the anionic head groups of phospholipids found in negatively charged membranes. Once these peptides bind to the membrane, their amphipathic helices can distort the lipid bilayer (with or without the formation of pores), resulting in the loss of membrane integrity. For example, polyphemusin I, a cationic antimicrobial peptide obtained from the horseshoe crab, has been shown to bind lipopolysaccharide coat of bacteria and permeabilize the outer membrane. However, it did not have any affinity for neutral lipids (10). The membranes of both prokaryotic cells and the mitochondria of eukaryotic cells maintain a large transmembrane potential and have a high content of anionic phospholipids, reflecting their common ancestry. In contrast, the plasma membranes of eukaryotic cells generally have a low potential and are composed almost exclusively of zwitterionic phospholipids (11). Many antibacterial peptides, therefore, preferentially disrupt prokaryotic and mitochondrial membranes (12, 13).

Tachyplesin, a small peptide composed of 17 amino acids, is isolated from the horseshoe crab (14). It has an amphipathic structure conferred by two antiparallel β-sheets held rigidly in place by two disulfide bonds. This structure seems to be critical for its antimicrobial activity (15). Previously, we found that a synthetic version of tachyplesin conjugated to the integrin homing domain RGD blocked the growth of tumor cells both *in vitro* and *in vivo* (5). RGD-tachyplesin inhibited the proliferation of both cultured tumor and endothelial cells and reduced the colony formation of TSU cancer cells. Importantly, *in vivo*, it could inhibit the growth of tumors via induction of apoptosis in both tumor and endothelial cells evidenced by activation of several caspases in both the mitochondrial and Fas-dependent pathways. More recently, we found that tachyplesin by itself (i.e., without the RGD domain) could inhibit tumor growth in the presence of normal serum even against cells that overexpress the multiple-drug resistant gene (data not shown).

However, little is known about the action mechanism of tachyplesin and it is unclear how it affects the plasma membranes of eukaryotic cells. The present study was to elucidate the mechanism by which tachyplesin was able to inhibit the growth of tumor and endothelial cells. Using the T7 phage display technique, we identified the C1q subcomponent of human complement 1 as a potential target of tachyplesin. The interaction between C1q and tachyplesin was confirmed by an ELISA and by affinity precipitation. The binding of tachyplesin to C1q seems to trigger the activation of the classic complement pathway and leads to the killing of TSU tumor cells. Interestingly, this effect was blocked if the target cells were pretreated with hyaluronidase or an excess of hyaluronan. This indicates that tachyplesin may initially

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target hyaluronan and related compounds on the cell surface and subsequently bind to C1q in the serum to activate the complement pathway. This represents a new mechanism for an antitumor agent.

Materials and Methods

Peptides and reagents. Two peptides were chemically synthesized: tachyplesin that consists of the sequence KWCFRVCYRGICYRRCR and a control peptide that contained the same amino acids but in a scrambled sequence. To impede enzymatic degradation of these peptides, the NH_2 terminals were acetylated and the COOH terminals were amidated.

Normal human sera were obtained from healthy volunteers. In some experiments, the serum was incubated at 56°C for 30 minutes to inactivate the complement system.

Reduction, alkylation, and acetylation of tachyplesin. The derivatization of the peptides was done as described by Prohaszka (7). A 1 mg/mL solution of tachyplesin in PBS was incubated with 20 mmol/L DTT at 37°C for 3 hours, followed by the addition of 60 mmol/L iodoacetamide at 4°C for 1 hour, which resulted in the complete reduction of disulfide bonds and the alkylation of the free -SH groups. The peptides were then dialyzed (molecular weight cutoff 1,000; Spectro/Pro) against water. Aliquots of the resulting peptides were further acetylated with acetic anhydride overnight. The reduced, alkylated, and acetylated peptides were thoroughly dialyzed against water, concentrated, and kept at −20°C until use.

Phage display. A T7 phage library of cDNA sequences from human breast tumor cells (T7 Select) containing 1.6×10^7 recombinants was purchased from Novagen (Madison, WI). Tachyplesin or control peptide (5 μg/mL) was coated onto Microtiter wells (Micro Membranes, Inc., Newark, NJ) overnight at 4°C and blocked with 1% bovine serum albumin. After washing, 2×10^9 plaque-forming units of the library were added to each well and incubated overnight at 4°C. The wells were then washed five times with 0.1% Tween 20 in TBS. The bound phages were recovered with 4 mol/L urea, diluted with TBS, and amplified in *Escherichia coli* in top agarose gel. After 3-hour incubation, the phage plagues were harvested for the next round of screening. This procedure was repeated four to five times until the plagues recovered in tachyplesin-coated wells outnumbered those in control peptide wells by 100-fold. Ten positive colonies were picked randomly and the DNA fragments encoding the binding protein were amplified by PCR using T7 Select Primer Up/Down pair and subjected to dideoxy sequencing. Eight of ten plaques gave an identical sequence. The deduced amino acid sequence was then used to search for their molecular identities and homologies using BLASTN 2.2.6 from Genbank + European Molecular Biology Laboratory + DNA Data Bank of Japan + Protein Data Bank sequence databases.

ELISA. In most cases, microtiter plates were coated overnight with human C1q protein or peptide diluted in coating buffer [0.1 mol/L Na_2CO_3/$NaHCO_3$ (pH 9.6)] and then blocked with 2% bovine serum albumin for 2 hours at room temperature. All subsequent steps were done in 1% gelatin in veronal-buffered saline (VBS) consisting of 5 mmol/L sodium barbital, 150 mmol/L NaCl, 0.15 mmol/L $CaCl_2$, and 1 mmol/L $MgCl_2$ unless otherwise indicated, and each step was followed by three washes with 0.1% Tween 20 in PBS. The enzymatic activity of peroxidase was assessed by the addition of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma, St. Louis, MO) and H_2O_2. The plate was read at a wavelength of 405 nm.

To confirm the binding of tachyplesin to C1q, two assays were done. First, the purified C1q (Sigma) were coated onto microtiter plates and the biotinylated tachyplesin was added at room temperature for 2 hours followed by peroxidase-conjugated streptavidin and ABTS substrate. Second, in reverse, the tachyplesin or the control peptide was coated and then C1q or normal human serum was added to the plate. The bound C1q was determined with a polyclonal rabbit antibody to C1q (1:1,000 dilution, DAKO, Carpinteria, CA) followed by a peroxidase-conjugated antirabbit IgG.

To detect the activation of the classic complement pathway, ELISA-based assays were done on plates coated with peptides (10 μg/mL) that were incubated with normal human serum diluted in VBS (C4b 1:2 for 30 minutes, C3b 1:2 for 20 minutes, and C5b-9 1:20 for 60 minutes) at room temperature according to their turnover rate (16, 17). The activated components were assessed using goat polyclonal antibodies to C4 (ICN, Aurora, OH) and to C3 and a rabbit polyclonal antibody to C5b-9 complex (Calbiochem, San Diego, CA).

Affinity precipitation and Western blotting. The serum samples (200 μL) were preabsorbed with 50 μL of streptavidin-Sepharose beads (Amersham, Piscataway, NJ) and then incubated with biotinylated tachyplesin (20 μg) and streptavidin-Sepharose beads (50 μL) overnight at 4°C. After washing, the bound proteins were eluted from the beads with 60 μL of Laemmli sample buffer (under reducing conditions) and electrophoresed on a 10% SDS-PAGE gel. After transfer to sheet of hydrophilic polyvinylidene fluoride (PVDF), the blot was immunostained with polyclonal antibodies to C1q (1:1,000) or to C4 (1:5,000) followed by a peroxidase-conjugated secondary antibody. The immunopositive bands were detected using Super Signal West Pico Chemiluminescent substrate (Pierce, Rockford, IL).

For the analysis of C3b deposition, TSU cells were incubated in 10% normal human serum along with tachyplesin or control peptide at 37°C for 60 minutes. After washing, the cells were harvested in lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 1 mmol/L EDTA, 0.5 mg/mL leupeptin, 1 mg/mL pepstatin, and 0.2 mmol/L phenylmethylsulfonyl fluoride). The protein concentration of the cell lysates was determined with the BCA reagent (Pierce). Twenty micrograms of protein from each lysate were electrophoresed on a 10% SDS-PAGE gel and then transferred to sheet of PVDF. The sheet was blocked in 5% nonfat milk and incubated with a polyclonal antibody to C3 (1 μg/mL) overnight at 4°C followed by a peroxidase-labeled secondary antibody and a chemiluminescent substrate as described above.

Fluorescence-activated cell sorting analysis. To analyze the attachment of tachyplesin to the cell surface, TSU tumor cells were harvested with 10 mmol/L EDTA and incubated in the presence or absence of 0.1 mg/mL testicular hyaluronidase (H4272, Sigma) for 1 hour at 37°C. FITC-tachyplesin (1 μg/mL) with or without 200 μg/mL of hyaluronan (Lifecore, Chaska, MN) was then added to the TSU cells and incubated at 4°C for 30 minutes followed by fixation with freshly prepared formaldehyde. The binding of FITC-tachyplesin to the cell surface was analyzed by flow cytometry.

For analysis of deposition of complement components on cell surface, TSU tumor cells were again harvested with EDTA and incubated in the presence or absence of 0.1 mg/mL testicular hyaluronidase for 1 hour at 37°C. Then, 100 μg/mL of the peptides dissolved in VBS was added to the cell cultures in the presence or absence of 10% normal human serum and the cells were incubated for 20 minutes at room temperature. The cells were washed, incubated sequentially with goat polyclonal antibody to human C4 or C3 (20 μg/mL, 30 minutes at 4°C), FITC-conjugated donkey anti-goat antibody (1:200, 30 minutes, at 4°C), and finally propidium iodide. The cells were analyzed by fluorescence-activated cell sorting (FACS) within 1 hour.

The permeability of the plasma membrane was analyzed by staining with FITC-dextran (M_r 40,000, Molecular Probes, Eugene, OR). For this, TSU cells grown to 80% confluence were incubated with tachyplesin (50 μg/mL) in the presence of 10% human serum overnight. After harvesting, the cells were incubated with 5 μg/mL of FITC-dextran for 30 minutes and then subjected to flow cytometry analysis.

Confocal microscopy. All cells were maintained in improved MEM medium supplemented with 10% FCS, 2 mmol/L L-glutamine, and antibiotics in 5% CO_2/95% air. For analysis of deposition of complement components on cell surface, TSU cells were seeded on coverlids at 50% to 60% confluence 18 to 24 hours before treatment and then incubated with tachyplesin (50 μg/mL) in the presence of 10% normal human serum for 20 minutes. After washing, a polyclonal antibody to C3 (1:200) was added to the cells at 4°C for 30 minutes followed by a FITC-labeled secondary antibody (1:200). After fixation in 4% formaldehyde, the coverslips were mounted using Prolong Antifade kit (Molecular Probes) and analyzed with a confocal microscopy.

To detect binding of tachyplesin to the cell surface, TSU tumor cells cultured on coverslips were incubated with FITC-tachyplesin (1 μg/mL) at

4°C for 30 minutes. After washing and fixating with 4% formaldehyde, the cells were examined with a confocal microscopy.

[^{3}H]Hyaluronan binding assay. The [^{3}H]hyaluronan (5×10^5 cpm/μg, 490 μg/mL) was isolated from the medium of rat fibrosarcoma cells grown in the presence of ^{3}H-labeled acetate as described previously (18, 19). Tachyplesin (50 μg) in 100 μL of PBS was mixed at room temperature for 2 hours with 20 μL of [^{3}H]hyaluronan in the presence or absence of a 100-fold excess of nonlabeled hyaluronan and then spotted onto a nitrocellulose membrane. The free [^{3}H]hyaluronan was washed away with PBS and the complex of [^{3}H]hyaluronan and tachyplesin that retained on the filter membrane was processed for scintillation counting.

Cell proliferation assay. The [^{3}H]thymidine incorporation assay was carried out as described previously (5, 20). Aliquots of complete medium containing ~10,000 cells were distributed into a 96-well tissue culture plate. On the next day, the media was replaced with fresh media without serum, peptide, and normal human serum or heat-inactivated serum. In some experiments, testicular hyaluronidase was added to fresh media before the addition of peptide and serum. One day later, 0.3 μCi of [^{3}H]thymidine in serum-free media were added to each well; after another 8 hours, the cells were harvested and the amount of incorporated [^{3}H]thymidine was determined with a β counter.

Trypan blue absorbance assay. To determine the extent of cell death, we used the trypan blue absorbance assay according to the method described by Uliasz and Hewett (21). For this, TSU tumor cells were subcultured into 24-well plates (1×10^5 cells/well) in 1 mL of 10% fetal bovine serum-90% DMEM. On the following day, the media was removed and cells were exposed to different concentrations of tachyplesin or control peptide in the presence of either native or heated-inactivated human serum. After 18 hours, a 0.4% solution of trypan blue was added to each well to a final concentration of 0.05% and further incubated at 37°C for 15 minutes. Next, media was removed and the cells were gently washed with ice-cold PBS (3×750 μL). The cells were then lysed with 200 μL of 1% SDS and gently triturated. Finally, 175 μL of the SDS/trypan blue solution was transferred to a 96-well plate and read spectrophotometrically at 590 nm. The background absorbance value was obtained from sham-treated cultures and was subtracted from all experimental values to obtain specific absorbance for each condition. All samples were assayed in triplicates.

Results

Isolation and characterization of tachyplesin-binding phages. To identify molecules that bind to tachyplesin, we screened a phage-displayed library of some 1.6×10^7 unique clones expressing sequences from breast cancer cells ranging in size from 300 to 3,000 bp in length fused to the *T7* gene 10 capsid protein. Phage particles expressing tachyplesin-binding proteins/peptides were affinity purified on the wells of a microtiter plate coated with tachyplesin or control peptide. After four or five rounds of biopanning, the number of phages from the tachyplesin-coated plates was ~100-fold greater than that from control peptide plates. Ten plaques were then selected and amplified by PCR. Eight had the same-size PCR products and some of these were then sequenced. The deduced amino acid sequences were then subjected to a blast analysis, which repeatedly identified human C1q. The region of C1q binding to tacyplesin was located around the first 400 bp of complement C1q B chain open reading frame (Genbank accession no. NM_000491), corresponding to the NH_2-terminal collagen-like domain of C1q (5, 22).

Binding of the C1q to tachyplesin. To further test the possibility that tachyplesin binds to C1q, we examined the interaction between these two proteins using an ELISA-like system. In the first assay, plates were coated with tachyplesin or the control peptide, probed with C1q, and then the amount of bound C1q was detected with anti-C1q. As shown in Fig. 1*A*, C1q binds to immobilized tachyplesin in a dose-dependent manner, but not to the control peptide. Similar results were obtained when the plates were precoated with C1q and then probed with biotinylated tachyplesin (Fig. 1*B*). However, this interaction was significantly reduced if the tachyplesin was denatured by reduction and alkylation of the disulfide bonds and further acetylation of the charged side chains (Fig. 1*C*), which suggests that the interaction between tachyplesin and C1q depends on the secondary structure of tachyplesin.

The binding of tachyplesin to C1q was also dependent on the NaCl concentration used in the assay buffer (Fig. 1*D*). The maximum binding occurred at 0.15 mol/L NaCl, the normal physiologic salt concentration. Both increasing and decreasing the ionic strength in the assay drastically reduced the binding.

The interaction between tachyplesin and C1q was further examined by affinity precipitation and Western blotting of normal human serum. For this, a biotinylated tachyplesin was incubated with normal human serum, followed by streptavidin-Sepharose. The immobilized proteins were then eluted and analyzed by Western blotting. As shown in Fig. 2, probing the blot with a polyclonal antibody revealed three bands corresponding to three chains of the C1q complex (*A*: 27.5 kDa, *B*: 25.2 kDa, and *C*: 23.8 kDa, respectively) in the tachyplesin-treated sample, but not in the samples without the peptide or with the control peptide. Not surprisingly, only a small portion of C1q from the serum was pulled down because serum contains a relatively high concentration of this protein (80 μg/mL; ref. 23). Taken together, these results suggests that C1q binds to both immobilized (surface-bound) and free (liquid-phase) tachyplesin, and tachyplesin binds to both purified and serum C1q, which confirms that there is true interaction between these two molecules.

Activation of the classic complement pathway by tachyplesin. To determine if tachyplesin could activate the complement pathway, we used ELISAs with tachyplesin-coated microplates. Normal serum was diluted with VBS (containing Ca^{2+}) and applied to wells coated with tachyplesin or the control peptide, washed, and then probed with antibodies against C4, C3, and C5b-9. As shown in Fig. 3*A*, significant amounts of activated fragments of C4b, C3b, and C5b-9 complex were bound to the immobilized tachyplesin but not to the immobilized control peptide. Furthermore, when the same fresh serum was heat-inactivated (56°C, 30 minutes) before use, then the amount of C4b, C3b, and C5b-9 complex was greatly reduced. These results show that tachyplesin is able to trigger the activation of whole classic complement cascade, which is characterized by the appearance of C4b, C3b, and C5b-9 complex.

To further test for the presence of activated C4b fragments, Western blotting was done. For this, normal fresh human serum was mixed with the biotinylated tachyplesin or control peptide, affinity-precipitated with streptavidin-Sepharose, and then subjected to SDS-PAGE and Western blotting using antibodies to C4. Figure 3*B* shows that the three peptide chains of C4b (α 97 kDa, β 75 kDa, and γ 33 kDa, respectively) were affinity-precipitated in the tachyplesin-treated sample, but not in those treated with the control peptide or in the absence of peptide. This is consistent with the results obtained from ELISA and further supports the conclusion that tachyplesin can activate the classic complement cascade. Whereas it is possible that tachyplesin binds directly to C4b, the phage display results suggest that this is not the case. Tachyplesin seems to initially and mainly bind to C1q and then to C4b.

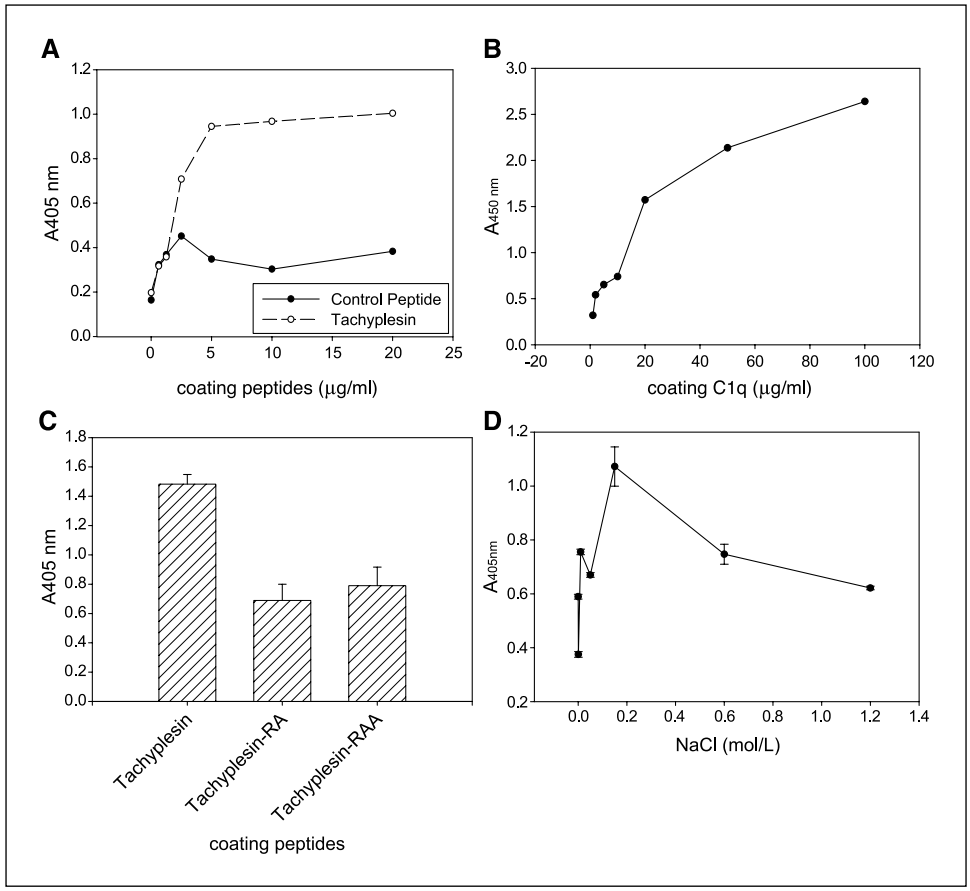

Figure 1. The interaction between tachyplesin and the C1q subcomponent of complement 1. *A,* ELISA plates were coated with varying concentrations of either tachyplesin or the control peptide and then incubated with 1 μg of C1q that was then detected with anti-C1q and peroxidase-conjugated detection system. The C1q bound to the plate coated with tachyplesin but not to that coated with the control peptide. *B,* plates were coated with varying concentrations of purified C1q, probed with biotinylated tachyplesin (1 μg/mL), and then assessed with the streptavidin-conjugated peroxidase and substrate system. Again, the tachyplesin bound to the immobilized C1q. *C,* similar amounts (10 μg/mL) of native, reduced, or alkylated (*RA*), and reduced, alkylated, and acetylated (*RAA*) tachyplesin replaced the tachyplesin in the above (*A*) binding system. The binding of C1q to native tachyplesin was significantly greater than either of the denatured peptides. *D,* to test the effect of ionic strength on the interaction, ELISA plate was coated with 10 μg/mL of C1q and then incubated with 0.1 μg/mL of biotinylated tachyplesin in VBS buffer of variable ionic strength. The maximum binding corresponded to physiologic ionic strength of 0.15 mol/L NaCl.

Role of hyaluronan in the deposition of C4b and C3b on tumor cells. Because tachyplesin contains a hyaluronan-binding motif [$B(X)_7B$; ref. 20], we investigated the possibility that tachyplesin can bind to hyaluronan (both free and cell associated). We took advantage of the fact that hyaluronan by itself does not bind to nitrocellulose but will do so in the presence proteins or peptides that bind to it (19). In the assay, tachyplesin was mixed with [^{3}H]hyaluronan and then applied to a nitrocellulose membrane. The free [^{3}H]hyaluronan was washed away and the complex of [^{3}H]hyaluronan-tachyplesin retained on the filter membrane was analyzed. Figure 4*A* shows that tachyplesin binds strongly to hyaluronan and this could be abolished by a 100-fold excess of unlabeled hyaluronan. In contrast, the control peptide showed little or no binding to [^{3}H]hyaluronan, indicating that the binding of tachyplesin to hyaluronan was specific.

We then examined the binding of FITC-tachyplesin to TSU cells that express large amounts of hyaluronan on their surfaces (20, 24). As shown in Fig. 4*B*, tachyplesin was distributed on the surface of the cells. This binding was significantly reduced by the addition of an excess of free hyaluronan on pretreatment with hyaluronidase (Fig. 4*C*) as shown by flow cytometry analysis. These results suggest that hyaluronan or related molecules, such as chondroitin sulfate (25), act as targets for tachyplesin on the cell surface.

In the classic pathway of complement activation, 4- to 5-fold more C3b than C4b is deposited on the surfaces of target cells (17). In addition, C3 contains a thioester moiety that can form covalent bonds with nearby molecules in the transition from C3 to C3b. Thus, C3b deposition represents an index of complement activation. For this reason, we tested whether tachyplesin could induce the deposition of C3b on tumor cells. TSU cells were incubated in a mixture of normal serum and tachyplesin, stained with antibodies to C3, then examined by confocal microscopy. As shown in Fig. 5*A* and *B*, C3b was indeed deposited on the surfaces of TSU cells.

The presence of activated C3b was also shown by Western blotting of TSU cells following treatment with serum and tachyplesin. Figure 5*C* shows that in the samples treated with

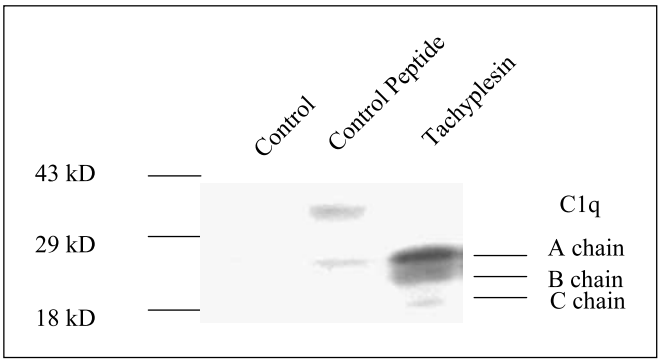

Figure 2. Affinity precipitation of serum C1q with tachyplesin. Normal human serum was incubated without or with biotinylated tachyplesin and control peptide, along with streptavidin-Sepharose in VSB buffer at 4°C overnight. After washing, the beads were extracted in Laemmli sample buffer (reducing conditions) and subjected to SDS-PAGE and Western blotting with anti-C1q antibody. Three bands representing the A, B, C chains of C1q (27,550, 25,200, 23,800 Da, respectively) were apparent in the sample incubated with biotinylated tachyplesin but not detected in the absence of peptide or in the control peptide. The results are representative of three different experiments.

tachyplesin, two subunits of C3b (α 115 kDa and β 75 kDa) and possibly degraded iC3b (? band for α1). Significantly, a high molecular weight band (*HMW* in Fig. 5*C*) was found with Western blotting under reducing conditions, indicating a covalent linkage to large membrane constituents. Scans of these Western blots revealed that the majority of deposited C3b (70-80%) was present in this high molecular weight form. This is consistent with the fact that in the transition from C3 to C3b, a thioester moiety, can form covalent bonds with nearby molecules. FACS analysis of cells treated with tachyplesin and serum (Fig. 5*D*) showed that there was a significant increase in FITC-tagged antibody to C3, indicating that C3b was deposited on the tumor cells, which did not occur with cells treated with the control peptide. These results are consistent with those from confocal microscopy and Western blotting.

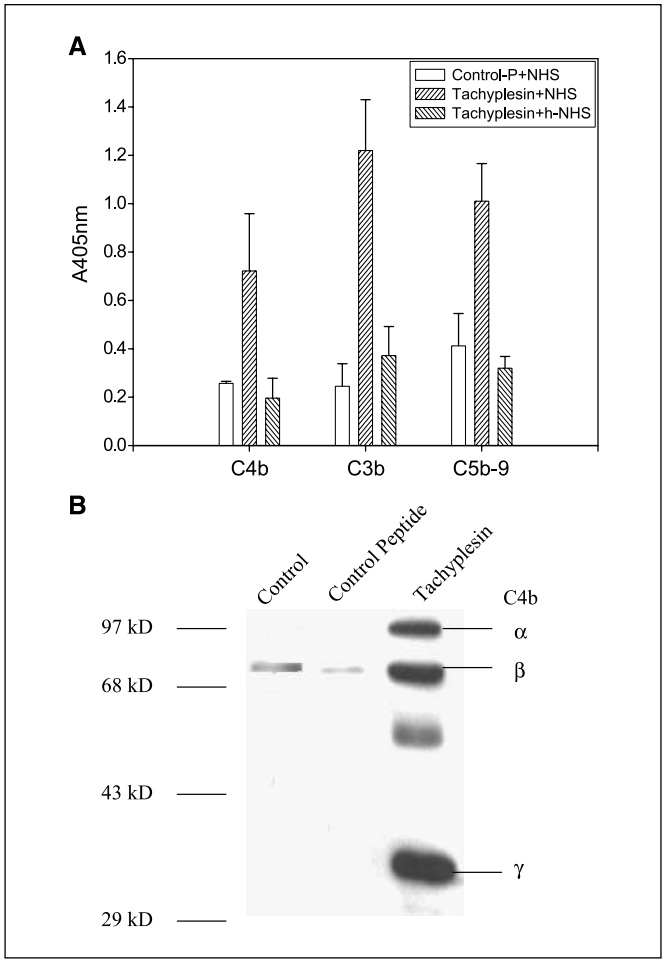

Figure 3. Determination of tachyplesin-mediated activation of the classic complement pathway by ELISA and affinity precipitation. *A,* microtiter plates were coated with either tachyplesin of the control peptide (10 μg/mL) incubated with normal human serum (*NHS*) without or with heat inactivation (*h-NHS*). The serum was diluted with VBS (1:2 for C4b, C3b and 1:20 for C5b-9). Complement activation was then assessed using polyclonal antibodies against C4, C3 and a polyclonal antibody to C5b-9. There was a significant amount of immobilized C4b, C3b and C5b-9 detected in the wells that were coated with tachyplesin and incubated with normal human serum compared with those coated with the control peptide or incubated with heat-inactivated serum. *B,* for affinity precipitation and Western blotting, normal human serum was treated without peptide or biotinylated control peptide or biotinylated tachyplesin. Streptavidin-Sepharose beads were added to the mixture, washed thoroughly, and processed for SDS-PAGE and Western blotting with antibodies to C4b. The bands α, β, γ chains of C4 (M_r 97, 75, and 33 kDa, respectively) were apparent in the samples treated with tachyplesin, but not with the control peptide or in the absence of peptide.

Because tachyplesin can bind to hyaluronan, we investigated the possibility that membrane-bound hyaluronan plays a role the binding of tachyplesin-mediated activation of complement on the surface of tumor cells. Suspensions of TSU cells were pretreated with hyaluronidase before the addition of tachyplesin and human serum; the presence of C3b was detected by immunostaining followed by FACS analysis. As shown in Fig. 5*D*, hyaluronidase pretreatment markedly reduced the intensity of fluorescence, indicating a reduction in C3b deposition. Thus, hyaluronan or related glycosaminoglycans seems to play a key role in the activation of complement on the cell surface by tachyplesin.

Effect of tachyplesin on tumor cells. The complement membrane attack complex (MAC, C5b-9) can damage the cell membrane, which results in the killing of the target cells. The possibility that tachyplesin can trigger the deposition of complement and the formation of C5b-9 on the surfaces of tumor cells via the classic pathway suggests that it might kill cells by disrupting the integrity of the plasma membrane. To test this possibility, we examined the permeability of cell membrane with macromolecule FITC-dextran, which is excluded by the membranes of viable, healthy cells but can pass through the damaged plasma membrane of unhealthy cells (2). Figure 6*A* showed that when cells were treated with tachyplesin and human serum, the fluorescence spectrum shifted, indicating that more FITC-dextran had passed through the plasma membrane and entered the cytoplasm. Thus, it seems that treatment with tachyplesin disrupted the cell membrane and increased its permeability.

Finally, we examined the effect of tachyplesin and normal human serum on tumor cell proliferation as measured by thymidine incorporation and cell death as measured by trypan blue uptake. As indicated in Figs. 6*B* and 7, TSU cells treated with tachyplesin in the presence of complete human serum showed a marked inhibition of proliferation and an increased extent of cell death. Figure 6*B* also showed that treatment with hyaluronidase significantly reversed the effects, again suggesting that cell-surface hyaluronan plays a critical role in tachyplesin-induced inhibition of tumor cell growth. Significantly, heat-inactivated serum also attenuated the effects of tachyplesin, but to a lesser extent than hyaluronidase treatment. These results imply that tachyplesin may have multiple effects on the cells leading to both growth arrest and death.

Discussion

The results of this study suggests the following multistep model to account for the antitumor activity of tachyplesin. First, tachyplesin binds to hyaluronan (or closely related glycosaminoglycans) on the surfaces of the target cells. Second, this bound tachyplesin interacts with C1q in the blood. Third, the tachyplesin activates the classic complement pathway. Finally, the formation of the MAC disrupts the integrity of the plasma membrane and results in the death of the target cells.

In the first step of our model, tachyplesin binds to the hyaluronan or related molecules on the surface of the target cells, is supported by several lines of evidence: (*a*) tachyplesin directly binds to soluble [^{3}H]hyaluronan, which may be due to the presence of a $B(X)_7B$ sequence that is known to bind hyaluronan (20); (*b*) pretreatment of TSU cells with hyaluronidase (which degrades both hyaluronan and chondroitin sulfate) resulted in a decreased tachyplesin-mediated deposition of C3b on the cell surface, leading to a decreased cytotoxic effects of tachyplesin as indicated by reduced [^{3}H]thymidine incorporation. It seems that the binding of

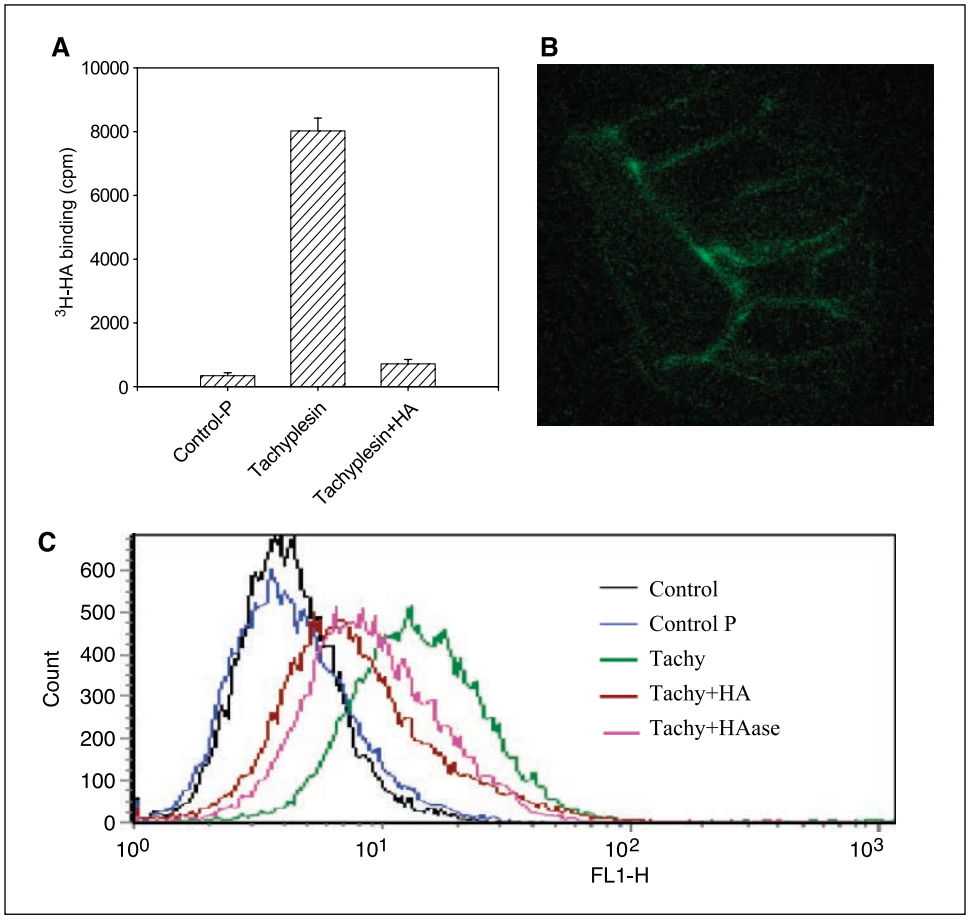

Figure 4. Hyaluronan-mediated attachment of tachyplesin to TSU tumor cells. *A,* to determine the binding of tachyplesin to hyaluronan, tachyplesin and the control peptide were mixed with [^{3}H]hyaluronan, applied to a sheet of nitrocellulose, and extensively washed. A significant amount of [^{3}H]hyaluronan bound to the nitrocellulose in the presence of tachyplesin but not in the control peptide or with an excess of unlabeled hyaluronan. *B,* 1×10^6 TSU tumor cells were incubated with 1 μg/mL of FITC-tachyplesin at 4°C for 30 minutes, fixed with freshly prepared 4% formaldehyde, and analyzed with confocal microscopy. Tachyplesin was associated with the surfaces of the cells. *C,* to determine the role of cell surface hyaluronan in the attachment of tachyplesin to tumor cells, the TSU cells were incubated with or without hyaluronidase (0.1 mg/mL) at 37°C for 1 hour. Then, 10 μg/mL of FITC-tachyplesin was added to the cells at 4°C for 30 minutes followed by fixation. Flow cytometry showed that the binding of FITC-tachyplesin to the cell surface was partially blocked by pretreatment with hyaluronidase or an excess of hyaluronan.

tachyplesin to cell surface hyaluronan is a critical first step in the subsequent induction of cytotoxicity. It is possible that in addition to hyaluronan, tachyplesin may also bind to chondroitin sulfate, which is also present on the surfaces of many cells (25) and is sensitive to digestion by testicular hyaluronidase. However, hyaluronan probably represents the major target because we had previously found that TSU cells have significant levels of cell surface hyaluronan (24).

The interaction between tachyplesin and hyaluronan could account for the relative specificity of antitumor activity of tachyplesin. Indeed, a body of studies has shown that many tumor cells (bladder, prostate, lung, colon, and breast) express high levels of hyaluronan whereas normal tissues express much less (26–28). In addition, studies have shown that endothelial cells involved in neovascularization express high levels of hyaluronan relative to those from established blood vessels (29). The fact that hyaluronan is preferentially expressed on the surfaces of tumors and endothelial cells involved in tumor vascularization could account for our previous observations that tachyplesin inhibits the growth of these cells relative to other nontumorigenic cells line that express less hyaluronan on their surfaces (5).

In the second step of our model, tachyplesin binds to C1q present in the serum. The phage display experiment reveals that tachyplesin binds to C1q, which is further confirmed by ELISA and affinity precipitation. The human C1q molecule (460 kDa) is a large complex composed of 18 polypeptide chains (6A, 6B, and 6C chains). As viewed under the electron microscope, the complex resembles a broccoli spear with common stalk that branches into six arms, each of which ends in globular head domains (22, 30). The phage display also reveals that tachyplesin binds to the stalk region of the B chain of C1q. This interaction seems ionic in nature because it was maximal under normal physiologic ionic strength and inhibited by either higher or lower ionic strength. However, the interaction clearly required the native configuration of tachyplesin that consists of a folded β-sheet stabilized by two disulfide bonds. When tachyplesin was reduced and alkylated, then the interaction with C1q was abrogated. In addition, the control peptide did not bind to C1q. Thus, whereas the interaction between tachyplesin and hyaluronan had an ionic component, the native structure of tachyplesin was essential for its activity.

In the next step of our model, the interaction of tachyplesin with C1q activates the classic complement pathway that consists of an enzyme cascade made up of numerous glycoproteins that under normal conditions exist in an inactive or proenzyme form. Activation of the complement system produces two major effects on the target cells: deposition of opsonic proteins (C3b and C4b) onto the cell surface and formation of C5b-9 (i.e., MAC). In this study, we have shown that the addition of fresh serum onto tachyplesin-coated plate results in the deposition of C1q downstream components C4b and C3b as well as the formation of C5b-9. In the case of C4b, this was confirmed by affinity precipitation followed by Western blotting. A similar process was found to occur on the cell surface. When TSU cells were treated with tachyplesin and serum, the C3b was deposited on their surfaces as determined by both fluorescent microscopy and

Western blotting. Importantly, heat inactivation of the serum, which destroys the complement system, also blocked many of the above processes, including the attachment of C4b, C3b, and C5b-9 on plastic plates coated with tachyplesin, and the deposition of C3b on the surfaces of TSU cells treated with tachyplesin. The ability of tachyplesin to induce the deposition of these downstream components strongly suggests the classic pathway of complement had been activated.

In the final step of our model, the formation of C5b-9 compromises the integrity of the plasma membrane leading to cell death. When cells were treated with tachyplesin and serum, there was an increased level of FITC-dextran entering the cytoplasm, suggesting the formation of large pores in the membrane, resulting in a decreased cell proliferation and viability. Significantly, this effect was diminished if the serum was heat-inactivated, again indicating that the activation of complement cascade was essential for cell damage effect of tachyplesin.

The formation of MAC is usually supposed to lyse the target cells. Indeed, several recent studies have indicated that activation of complement and the formation of the MAC can also result in apoptosis of target cells. For example, Nauta et al. (31) have shown that C5b-9 induces apoptosis through a caspase-dependent pathway that can be blocked by the inhibitor zVAD-fmk. Similarly, Cragg et al. (32) reported that activated complement results in DNA fragmentation, a characteristic of apoptosis. Our previous study showed that RGD-tachyplesin could induce apoptosis in a number of target cells (5) by increasing the Annexin V staining, activation of caspases 9, 8, and 3, and increase of the expression of the Fas ligand, Fas, caspase 7, and caspase 6. Based on these results, we had proposed that tachyplesin could activate apoptotic molecules involving both mitochondrial and Fas-dependent pathways. The results of the present study indicate that at least some of this apoptosis resulted from the compromising of the plasma membrane due to the tachyplesin-mediated activation of the classic complement cascade.

Whereas our model for the antitumor activity of tachyplesin is consistent with all of our previous data, it must be acknowledged that tachyplesin may have additional effects. We had found that even when the complement system was heat-inactivated, there was still some residual antitumor activity implying that other mechanisms may be involved. It is possible that even in the absence of the complement system, tachyplesin is able to directly compromise the plasma membrane, as it is believed to do with microbes.

Our results also indicate that tachyplesin is similar to other nonimmune activators of the classic complement pathway in that it binds to the stalk-like region of C1q, in contrast to the immune activators (IgG and IgM) that bind to the globular regions (30, 33, 34). It should be noted that simply binding to C1q is not sufficient to activate the complement cascade that is controlled at many levels as a safeguard against unintended activation by the

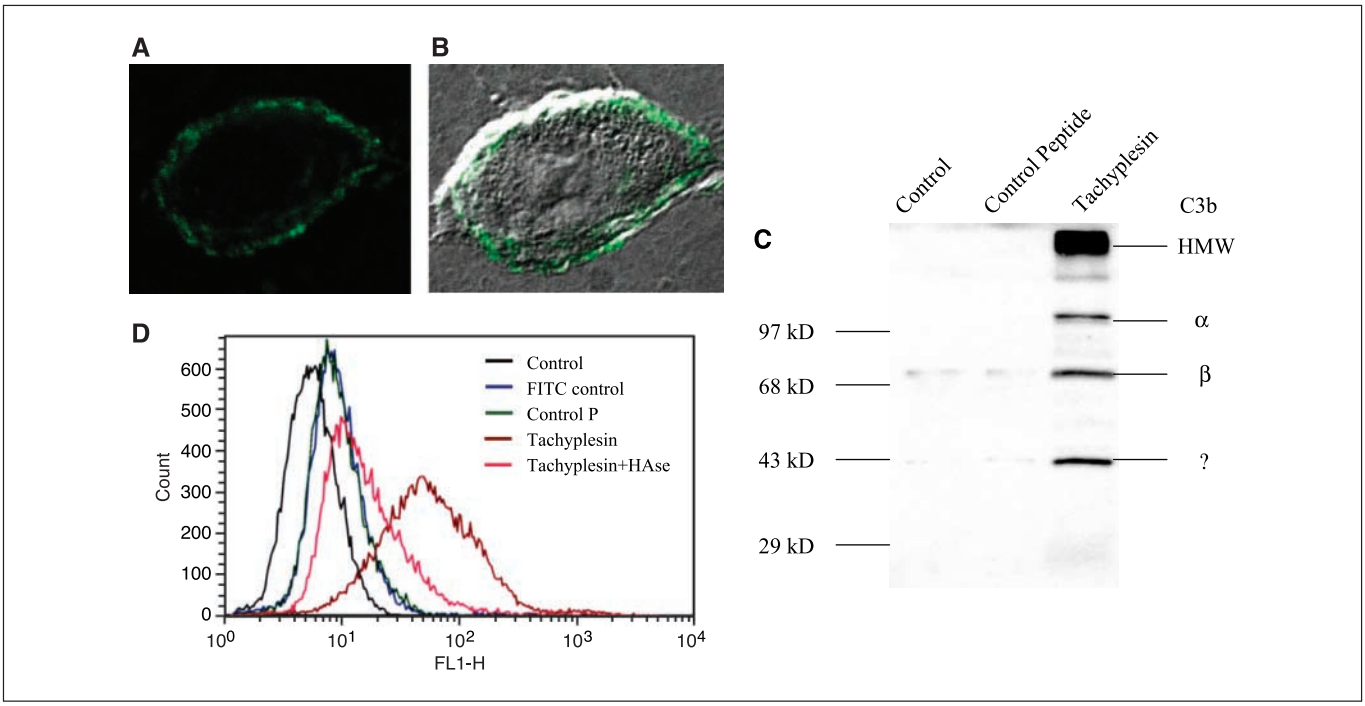

Figure 5. Effect of tachyplesin on the induction of complement on the surfaces of tumor cells. *A* and *B,* TSU cells were incubated with tachyplesin in the presence of 10% normal human serum for 20 minutes, washed, and then incubated with a polyclonal antibody to C3 (4°C for 30 minutes) followed by a FITC-secondary antibody and analysis by confocal microscopy. Both immunoflourescence alone and the merger of fluorescence and transmitted images are shown. *C,* to analyze complement activation by Western blotting, TSU cells were incubated in 10% normal human serum in the presence or absence of either tachyplesin or control peptide at 37°C for 60 minutes. After washing, the cell lysates were harvested and processed for Western blotting with antibodies to C3b. In the cells treated with tachyplesin, there were two bands corresponding to α and β subunits of C3b (115 and 75 kDa), as wells as a high molecular weight band representing C4b covalently bound to membrane constituents. These bands were reduced in cells treated without peptide or with the control peptide. *D,* to examine the role of cell surface hyaluronan in the deposition of complement, 100 μg/mL peptides were added to the cells culture in the presence of 10% normal human serum and incubated for 20 minutes at room temperature. The cells were then washed, incubated with a polyclonal antibody to human C3 (20 μg/mL) at 4°C for 30 minutes, followed by incubation with FITC-conjugated IgG (1:200) at 4°C for 30 minutes. The cells were finally stained with propidium iodide and analyzed with FACS. Preincubation with hyaluronidase abrogated the C3 deposition on TSU cell surfaces.

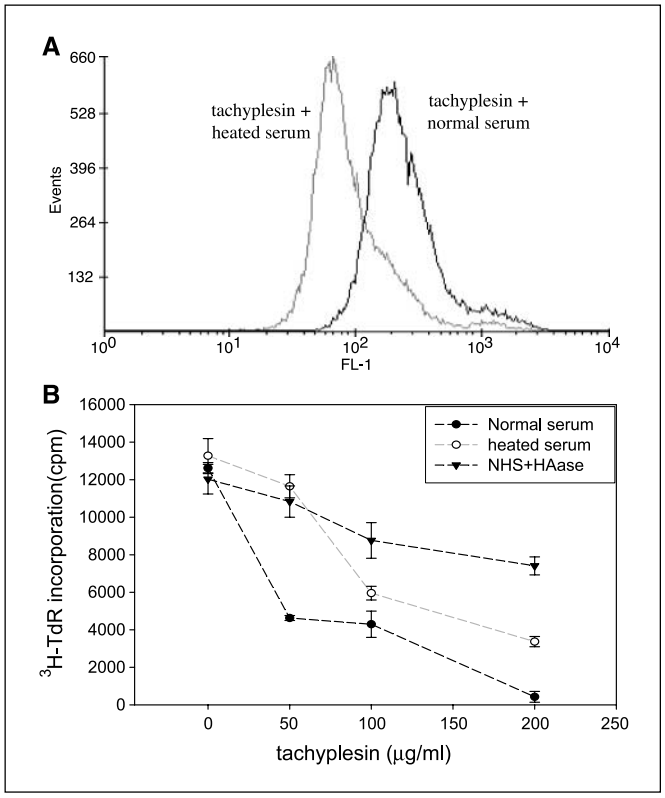

Figure 6. Biological effect of hyaluronidase on tachyplesin-mediated cytotoxicity of tumor cells. *A,* to determine whether tachyplesin damages the plasma membrane, TSU cells were incubated with tachyplesin plus heat-inactivated human serum or normal human serum overnight. After harvesting, the cells were incubated with 5 μg/mL of FITC-dextran (M_r 40,000) and subjected to flow cytometry. The amount of FITC-dextran taken up by the cells was significantly higher in those treated with tachyplesin than in the control cells, suggesting that tachyplesin disrupts the integrity of the plasma membrane. *B,* TSU tumor cells were treated with hyaluronidase (0.1 mg/mL) at 37°C for 1 hour. Then, tachyplesin and 2% normal human serum or heat-inactivated serum were added and incubated overnight followed by a [^{3}H]thymidine incorporation assay. The cytotoxic effects of tachyplesin in present of normal serum were reduced by either treatment with hyaluronidase or heat-inactivated serum ($P < 0.05$).

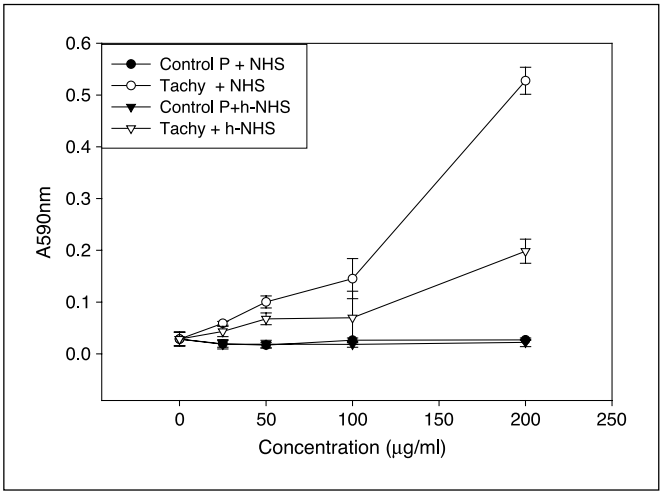

Figure 7. Cell death induced by tachyplesin and its activated complement. TSU tumor cells were cultured in 24-well plates and then exposed for 18 hours to different concentrations of tachyplesin or control peptide (*Control P*) in the presence of either normal or heat-inactivated human serum. Then, a solution of 0.4% trypan blue was added to each well to a final concentration of 0.05%. The cells were incubated at 37°C for 15 minutes, washed, and lysed in 200 μL of 1% SDS. The amount of dye taken up by the cells was determined by reading the absorbance at A_{590}. All samples were assayed in triplicates. The cells treated with tachyplesin and normal human serum took up a significantly greater amount of dye compared with those treated with control peptide or heated-inactivated serum ($P < 0.05$).

body. Indeed, there are numerous modifiers in both the serum and on the cell surface that regulate the complement cascade (35, 36). Several studies have shown that *in situ* tumor cells overexpress some of these membrane-bound regulatory proteins, which may down-regulate an efficient local immune response, such as those induced by monoclonal antibody-mediated immunotherapy (37, 38). Tachyplesin seems to be able to bypass these "protective" mechanisms for tumor cells.

In conclusion, we believe that the model proposed for the antitumor activity for tachyplesin represents a new and promising avenue for targeting and attacking tumor cells. Previously, we had found that tachyplesin is capable of preventing tumor growth in both mice and chicken embryos without significant side effects (5). However, the concentration required to achieve a therapeutic effect was relatively high. If our proposed model is correct, then it may be possible to design other molecules with improved therapeutic effects in the way that one subdomain could be dedicated to the binding of hyaluronan and another to the binding and activation of C1q. At the same time, these peptides could be modified in such a way as to increase their stability and half-life in circulation. Such compounds could be effective as a new agent in a multipronged attach of tumor cells.

Acknowledgments

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