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Effect of Exosomal Nanoencapsulated Alab™ peptide on the IL-8, COX-2, NF-kB, and Angiogenesis *in vitro/in vivo* Against Human Gastric Cancer for Nutrient Delivery System

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ABSTRACT
Because nuclear factor-kB (NF-kB) has been implicated in the pathogenesis of gastric carcinoma, this transcription factor is a potential target for the treatment of this devastating disease. Alab™ peptide is one of the antitumor peptides derived from *Lactobacillus helveticus* KTCT 15075BP, and it has shown NF-kB inhibitory activity. It is pharmacologically safe, but its bioavailability is poor after oral administration. Alab peptide was nanoencapsulated in a exosomal delivery system to allow intravenous administration of smart probiotic Nutrient Delivery System(NDS). We studied the *in vitro* and *in vivo* effects of this exosomal nanoencapsulated alab peptide on proliferation, apoptosis, signaling, and angiogenesis using human gastric carcinoma cells. NF-kB was constitutively active in all human gastric carcinoma cell lines evaluated, and exosomal nanoencapsulated alab peptide consistently suppressed NF-kB binding and decreased the expression of NF-kB-regulated gene products, including cyclooxygenase-2 and interleukin-8, both of which have been implicated in tumor growth/invasiveness. These *in vitro* changes were associated with concentration and time-dependent antiproliferative activity and proapoptotic effects. The activity of exosomal nanoencapsulated alab peptide was equal to or better than that of free alab peptide at equimolar concentrations. *In vivo*, alab peptide suppressed gastric carcinoma growth in murine xenograft models and inhibited tumor angiogenesis. Exosomal nanoencapsulated alab peptide down-regulated the NF-kB machinery, suppressed growth, and induced apoptosis of human gastric cells *in vitro*. Antitumor and anti-angiogenic effects were observed *in vivo*. The experiments in the current study provide a biological rationale for systemic treatment of patients suffering from gastric carcinoma with this nontoxic probiotic chemical nanoencapsulated in exosomes as a smart probiotic NDS.

KEYWORDS: gastric carcinoma, apoptosis, angiogenesis, exosomal nanoencapsulation, probiotic Nutrient Delivery System(NDS), anticancer effects, Alab™ peptide

I. INTRODUCTION

Recently, exosomal nanoencapsulation technology combines nanotechnology and biotechnology in a very powerful way. Medical specialists have used exosomal nanoencapsulation technology for many years to deliver tiny amounts of therapeutic substances to specific organs or tissues without alteration and without affecting any other parts of the body. Many health-promoting substances can be nanoencapsulated in sub-microscopic exosomes. The exosomes not only protect the supplement from degrading conditions in the environment, but will also deliver them safely to "target" organs and structures of the body.[1-3] We have recently perfected a smart probiotic Nutrient Delivery System(NDS) called Alab™ using “smart exosomal nanoencapsules”-a exosomal encapsulation technology developed especially for NDS.[4-8] NDS is proposed to increase the efficacy and safety of nutrients [5,9,10] Since 1993, various approaches have been developed considering nanoparticles as NDS.[5,11-13] Using the SCF process, exosomes, designated as critical fluid exosomes (CFE), encapsulating hydrophobic drugs, such as taxoids, camptothecins, doxorubicin, vincristine, and cisplatin, were prepared. Also; stable paclitaxel exosomes with a size of 50 to 100 nm were obtained.

Aphios Company's patent (US Patent No. 5,776,486) on Super Fluids™ CFL describes a method and apparatus useful for the nanoencapsulation of paclitaxel and campothecin in aqueous exosome formulations called Taxosomes™ and Camposomes™, respectively. These formulations are claimed to be more effective against tumors in animals compared to commercial formulations.[14]

Probiotics are defined as live microbial food components that are beneficial for human health. Recently, because they exhibit beneficial effects equal to those of live microbes, genetically engineered microbes and nonviable microbes have been regarded as probiotics.[15,16] Lactic acid bacteria, one of the most common types of probiotic bacteria, have been reported to exhibit beneficial effects on host homeostasis, including activation of immune function.[17,18] Probiotic *Lactobacillus helveticus* reduces diarrhea[19,20] and appears to modify the digestive microflora[21,22] and to enhance the immune system during its transit in the digestive tract.[23-25]

Gastric carcinoma is an aggressive, chemotherapy-resistant malignancy. As the 5th leading cause of cancer-related mortality, it remains a major public health problem, accounting for an estimated 30,000 deaths per year in the U.S.[26] Surgical resection is the only curative therapy, but, to the best of our knowledge, virtually no patients survive 5 years. Gemcitabine, approved by the Food and Drug Administration in 1996, is the only chemotherapeutic agent licensed for use in gastric carcinoma. However, gemcitabine results in an objective tumor response in approximately 5% of patients and the impact on survival is minor [27] That it is considered the most active agent in this disease, despite such limited efficacy, highlights the desperate need for new therapeutic strategies.

Epidemiological and animal studies have shown that the microchemicals present in the diet can be effective agents for the prevention of cancer.[28-51] Some of these compounds have significant antitumor activity *in vitro* and in preclinical models. Due to the lack of toxicity, there is increasing interest in probiotic culture fluid that exhibits such activity. The possibility of using biochemicals with established biopreventive activities and preclinical antitumor effects as a novel approach for management of established cancer merits exploration.

Alab™ peptide, an antitumor peptide isolated from *Lactobacillus helveticus* KTCT 15075BP, is a food supplement and pharmacologically safe as indicated by its consumption as a natural probiotic NDS.[4,6-8,14,31,32] The molecular mass of alab peptide is 4.5kDa and is made of 44~46 amino acid residues. The N-terminal peptide sequence of alab peptide was given as follows: Ala-Ile-Ala-Leu-Val-Ala. In our previous in vitro study, alab peptide at 100 mg/mL inhibited the growth of three colon cancer cells (CHT-29, SW 480, and Caco-2) over 70%. The results showed that alab peptide decreased cell viability in a concentration-dependent manner. The physiological significant of lactic acid bacteria induced cytokine secretion to human health remains to be clarified TNF-α exerts cytotoxic effects on tumor cells.[31]

This has potent antiproliferative and proapoptotic effects *in vitro*.[33-37] In murine models, alab peptide suppresses carcinogenesis of the skin,[38-41] the bladder,[42-45] the breast,[46,47] and the colon[31, 50,51], as well as rheumatoid arthritis,[48,49] in mice.

Because alab peptide has diverse effects on signaling molecules relevant to cancer, multiple mechanisms of action could account for its antitumor effect. Importantly, it is a potent inhibitor of NF-kB, a transcription factor implicated in the pathogenesis of several malignancies including gastric carcinoma.[52] NF-kB-regulated genes include IkBα, cyclooxygenase-2 (COX-2), and interleukin-8 (IL-8).[53,54] Gastric carcinoma cells often express high levels of IL-8 and COX-2, and these molecules appear to play a role in the proliferative and metastatic potential of this neoplasm.[55-59] Therefore, suppression of NF-kB and its downstream effectors may be an innovative strategy for treatment of gastric carcinoma.

In the current study, we investigated the *in vitro* and *in vivo* antitumor activity of exosomal nanoencapsulated alab peptide as a smart probiotic NDS against human gastric carcinoma cells. Our results demonstrate that all six gastric carcinoma cell lines used in this study expressed constitutively active NF-kB, which was suppressed by exosomal nanoencapsulated alab peptide. NF-kB inhibition was associated with growth suppression, apoptosis, and down-regulation of expression of gene products (IL-8 and COX-2) regulated by NF-kB. *In vivo*, exosomal nanoencapsulated alab peptide inhibited gastric carcinoma growth and exhibited anti-angiogenic effects.

II. MATERIALS AND METHODS

Cell Lines

The human gastric carcinoma cell lines BxPC-3, Capan-1, Capan-2, ASPC-1, HS766-T, and MiaPaCa2 were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured according to the supplier's instructions.

Materials

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and (DMPC/DMPG) (DMPG = 1,2-dimyristoyl-sn-

glycero-3-[phospho-rac-(1-glycerol)] [sodium salt]) were obtained as dry powder from Avanti Polar Lipids (Alabaster, AL)., dimethylsulfoxide (DMSO), acetone, and tert-butanol were obtained from Sigma Chemical Company (St. Louis, Mo, USA). Alab™ peptide from probiotic *Lactobacillus helveticus* KTCT 15075BP was kindly provided as dry powder by KimJungMun Aloe Co. Ltd. (Jeju-do, Korea).

Exosome Preparation

We tested various total exosome : alab peptide ratios (weight/weight) ranging from 10:1 to 4:1 before deciding to use a fixed ratio of 10:1 in subsequent experiments. The lyophilization procedure involved several steps. First, alab peptide was dissolved in 50 mg/mL DMSO. The lipid (e.g., DMPC) was dissolved in 20 mg/mL tert-butanol. The two solutions were mixed and filtered through a 0.22-μM filter (Millipore, Concord Road, MA, USA) for sterilization. Aliquots of this solution were placed in lyophilization vials. The vials were frozen in a dry ice/acetone bath and lyophilized for 24 hours to remove all DMSO and tert-butanol. The vials were stored at -20°C. The lipid formulation included DMPC or DMPC/DMPG.

MTT Cell Proliferation Assay

Proliferation/survival of cells after exosomal nanoencapsulated alab peptide exposure was assessed by the MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide) assay according to the manufacturer's instructions (Sigma-Aldrich Company, USA). Cells were incubated for 72 hours (0 hr, 12 hrs, 24 hrs, 48 hrs, and 72 hrs, respectively). The mean value and standard error for each treatment were determined, and then converted to percent relative to control (empty nanosized exosomes). The concentration at which cell growth was inhibited by 50% (IC_{50}) was determined by linear interpolation using the formula [(50% - low percentage)/(high percentage - low percentage)] x (high concentration-low concentration) + low concentration. IC_{90} was similarly determined, with 90% substituting for 50% in the above equation.

Cell Recovery Assay

To determine whether cells treated with exosomal nanoencapsulated alab peptide recovered their proliferative capacity after removal of alab peptide, the cell recovery assay was used. Cells were seeded at 5.0 x 10^5 cells per 100-mm plate (BD Biosciences , San Jose, CA, USA) and incubated at 37℃ in a humidified atmosphere with 5% CO_2 overnight. Cells were treated with various concentrations of exosomal nanoencapsulated alab peptide and incubated for 72 hours. Cells were trypsinized and counted, and the same number of cells for each treatment (3.0-5.0 x 10^3 cells per well, depending on the cell line) were then seeded in triplicate into 96-well plates in alab peptide-free medium and incubated for 72 hours. The degree of cell recovery was determined via MTT assay.

Annexin V/Propidium Iodide Staining for Apoptotic Cells

Cells were seeded at 2.5-5.0 x 10^5 cells per 100-mm plate, depending on the cell line, and incubated at 37℃ in a humidified atmosphere with 5% CO_2 overnight. After they were grown to confluence in 75-cm^2 tissue culture flask (Nunk, Roskilde, Denmark), cells were detached and transferred to in new cell culture dishes using trypsin-versene mixture (Cambrex Bio Science, Thomaston Street Rockland, ME, USA) for each experiments. Cell number and viability were assessed by trypan blue dye exclusion on a Neubauer hemocytometer (American Optical, Buffalo, NY, USA). Cells were treated with various concentrations of exosomal nanoencapsulated alab peptide or free alab peptide in DMSO (the final DMSO concentration was 0.1%) and incubated for 72 hours. The cells were harvested by quick (< 5 minutes) trypsinization to minimize potentially high annexin V background levels in adherent cells. Cells were then washed and stained with fluorescein 5(6)-isothiocyanate (FITC)/annexin V/propidium iodide (PI) as directed by the annexin V-FLUOS staining kit (Roche Diagnostics, Indianapolis, USA). Stained cells were placed on ice and protected from light until read via flow cytometry. Cells were analyzed on an Epics XL-MCL flow cytometer using the System II version 3.0 software (both hardware and software are from Beckman Coulter, Miami, FL, USA), with the laser excitation wavelength set at 488 nm. The green signal from FITC/annexin V was measured at 525 nm and the red signal from PI was measured at 620 nm. Cells staining negative for both annexin V and PI are viable. Cells that are annexin V+/PI- are in early apoptosis, and cells that are necrotic or in late apoptosis are annexin V+/PI+ as previously described.[18,19]

Electrophoretic Mobility Shift Assay for Nuclear Factor-kB

Electrophoretic mobility shift assay (EMSA) was performed using standard procedures and was used to detect NF-kB binding before and after exosomal nanoencapsulated alab peptide treatment. Cells seeded at 5.0 x 10^5 cells per 100 mm plate and incubated overnight were treated with IC_{50} concentrations of exosomal nanoencapsulated alab peptide and incubated for 72 hours.

Nuclear extracts were thawed on ice, and protein concentration was determined by the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). For supershifts, 2 μg of rabbit polyclonal anti-p50 or p65

antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added to the nuclear extract and incubated at room temperature for 5 or 15 minutes before its addition in the binding reaction.
Autoradiographs were quantified on the Fluorchem 8900 imaging system using Alpha Ease FC software (Alpha Innotech, San Leandro, CA, USA).

Oligonucleotides
The consensus sequence wild-type and mutant blunt-end double-stranded oligonucleotides used for the NF-kB EMSAs were purchased from Santa Cruz Biotechnology. The NF-kB wild-type sense strand sequence was 5'-AGTTGAGGGGACTTTCCCAGGC-3' and the mutant sequence was 5' -GTTGAGGCGACTTTCCCAGGC-3'. Oligonucleotides were ^{32}P-end-labeled with T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA), purified with Quick Spin G-50 Sephadex columns (Roche Diagnostics, Rotkreuz, Switzerland), and stored at -20°C.

Interleukin-8 Enzyme-Linked Immunosorbent Assay
Levels of IL-8 protein in conditioned media before and after exosomal nanoencapsulated alab peptide exposure were determined by enzyme-linked immunosorbent assay (ELISA) using the Quantikine human IL-8 immunoassay kit by R & D Systems (Minneapolis, MN, USA). The lower limit of sensitivity for the assay was 10 pg/mL.

Immunoblotting
Immunoblotting using standard procedures was performed to assess steady-state levels of COX-2 protein and to determine polyadenosine-5'-diphosphate-ribose-polymerase (PARP) cleavage (the latter reflecting apoptosis). Protein content was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce Endogen, Rockford, IL, USA). The samples were run on 6-10% sodium dodecyl sulfate gels. The signals were detected by secondary antibodies (horseradish peroxidase-conjugated anti-mouse immunoglobulin [Ig] or anti-rabbit Ig, as appropriate [1:5000]) (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and the enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and then autoradiographed.

Antibodies
Antibodies used for immunoblotting included monoclonal anti-tubulin antibody (Sigma Chemical Company, Spruce St., MO, USA), anti-PARP rabbit polyclonal (Cell Signaling, Beverly, MA, USA), and anti-COX-2 monoclonal antibody (MoAb) (Cayman Chemical, Ann Arbor, MI, USA). For EMSAs, rabbit polyclonal anti-p50 and anti-p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. Anti-vascular endothelial growth factor (anti-VEGF) MoAb was obtained from Oncogene (Boston, MA, USA) and rat anti-mouse CD31 from BD Pharmingen (San Diego, CA, USA).

Animals
Female athymic nu/nu Sprague-Dawley mice (3-5 weeks old) obtained from Daehan Laboratory Animal Research (Seoul, Korea) were maintained 5 per cage in microisolator units. Animals were given a commercial diet and water. Mice were quarantined for ≥ 1 week before experimental manipulation. All animal experiments were performed at the R&D Center of KJMbio Co. Ltd. (Seoul, Korea) under protocol 10-02-13231 approved by the Institutional Animal Care and Use Committee of the Jeju National University animal laboratory.

Animal Models
A total of 5 x10^6 BxPC-3 or MiaPaCa2 cells collected in 100 μL RPMI media in log phase growth were injected subcutaneously on 1 side of the abdomen of 3-5 week-old female nude mice. Once tumor masses were established, animals were randomized to receive intravenous exosomal nanoencapsulated alab peptide (intravenous tail vein; 40 mg/kg body weight, 3 times per week; this is the maximum volume that could be injected), empty exosomes, or saline. Tumor size and body weight were measured with calipers three times a week. Tumor volume was calculated using the following formula: volume = (length x width^2)/2, in which width was the shortest measurement in millimeters.

Immunohistochemistry for Angiogenesis
Immunohistochemistry studies were performed with formalin-fixed, paraffin embedded sections (5μm), heat-induced antigen retrieval (Dako Corporation, Carpinteria, CA, USA), and 1:200 monoclonal anti-VEGF antibody (Oncogene) or 1:50 rabbit polyclonal anti-IL-8 antibody (Biosource International, Camarillo, CA, USA). The frozen sections were cut from snap-frozen tissue and embedded in OCT compound (Miles, Elkhart, IN, USA). Separate sections were incubated with 1:200 rat anti-mouse CD31 antibody (BD Pharmingen, San Diego, CA, USA). The detection system was the LSAB2 detection kit (Dako Corporation, Santa Barbara, CA,

USA). The secondary antibody was a biotinylated antibody (Dako Corporation, Santa Barbara, CA, USA) that forms a complex with peroxidase-labeled streptavidin. Sections were counterstained with Gill's hematoxylin (Sigma Chemical Company, St. Louis, Mo, USA). For the negative controls, the primary antibody was replaced with anti-IgG1 (the same isotype as the primary antibody) (Oncogene).

Statistical Analysis

The experimental results are expressed as means ± the standard errors. The data was compared using one-way analysis of variance (ANOVA) followed by Duncan post-hoc test to determine statistical differences after multiple comparisons (SAS; SAS Institute Inc., NC, USA). *P*-values < 0.05 were considered statistically significant.

III. RESULTS

We used the human pancreatic cell lines ASPC-1, BxPC-3, Capan-1, Capan-2, HS766-T, and MiaPaCa2 to investigate the effect of exosomal nanoencapsulated alab peptide on cell proliferation and apoptosis and on constitutive NF-kB activity and NF-kB-regulated gene expression.

Exosomal nanoencapsulated alab peptide Inhibits Proliferation/Survival of Gastric Carcinoma Cell Lines *In Vitro*

Exposure to exosomal nanoencapsulated alab peptide (for 0 hr, 12 hrs, 24 hrs, 48 hrs, and 72 hrs, respectively) inhibited gastric cell growth in all 6 lines tested in a concentration- and time-dependent manner (Fig. 1). Proliferation/survival was assessed by MTT assay. MTT is a pale yellow substrate that is cleaved by living cells to yield a dark blue formazan product. This colorimetric change reflects active cell proliferation/survival. IC_{50} concentrations varied from approximately 2.0 μM for Capan-1 to 37.8 μM for Capan-2. The IC_{50} for free alab peptide ranged from 5.4 μM (BxPC-3 and Capan-1 cells) to 46 μM (Capan-2 cells). The antiproliferative effects of exosomal nanoencapsulated alab peptide were equivalent or better than those of free alab peptide at equimolar concentrations in all cell lines ($p < 0.05$).

Growth-Inhibitory Effects of Exosomal Nanoencapsulated Alab Peptide Are at Least Partially Irreversible

After exposure to exosomal nanoencapsulated alab peptide for 72 hours, gastric carcinoma cells were replated in fresh media, and recovery of proliferation/survival was assessed by MTT assay after an additional 72 hours. The concentrations of exosomal nanoencapsulated alab peptide were approximately the IC_{50} and IC_{90} for each cell line. There was a concentration-dependent loss of ability to recover in all six cell lines, although this was not complete in all cell lines ($p < 0.05$). These results suggest that the cells had at least partially undergone irreversible changes such as apoptosis (Fig. 2A; BxPC-3, 2B; HST66-T, and 2C; MiaPaCa-2).

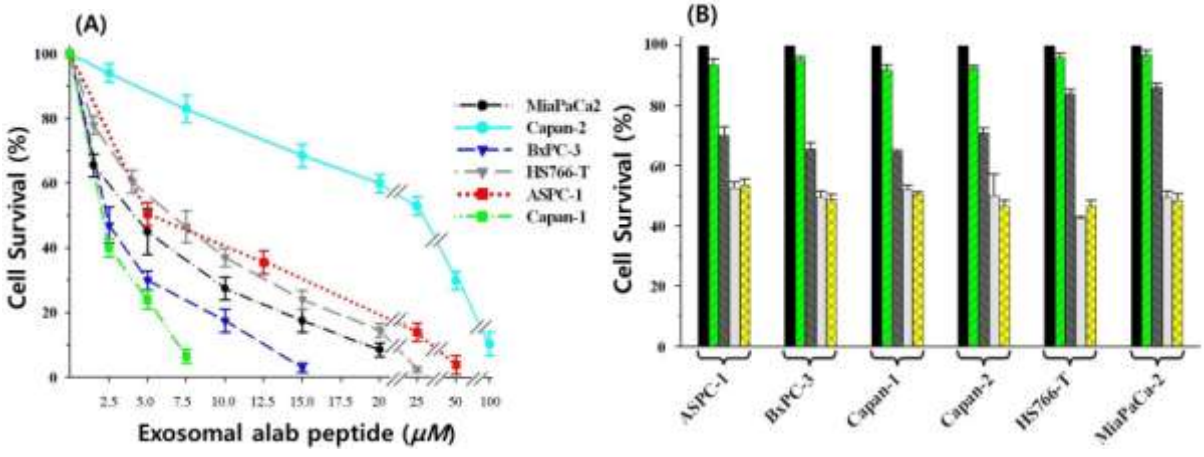

Figure 1. Effect of exosomal nanoencapsulated alab peptide on proliferation/survival as assessed by the MTT assay. Exosomal nanoencapsulated alab peptide inhibits the growth of all six gastric cell lines tested. Values represent the mean ± standard error of triplicate experiments after 72-hour exposure to exosomal nanoencapsulated alab peptide ($p < 0.05$). (A) Dose-dependent inhibition. (B) Time-dependent inhibition of cells exposed to IC_{90} levels of alab peptide (as determined in panel A). IC_{90}: concentration at which cell growth was inhibited by 90%. ■ ; 0 hr, ▨ ; 12 hrs, ▨ ; 24 hrs, □ ; 48 hrs, and ▩ ; 72 hrs. *$p < 0.05$ when compared with control cells.

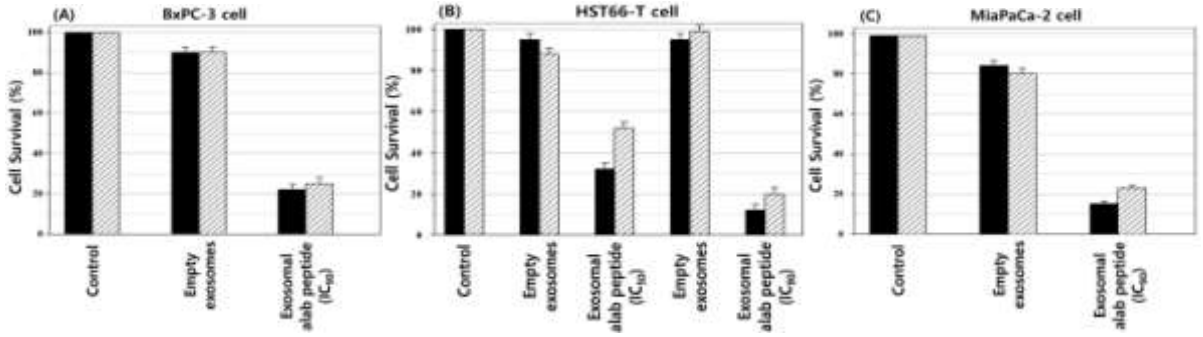

Figure 2. (A-C) Failure of proliferation/survival (MTT assay) to recover after treatment with exosomal nanoencapsulated alab peptide. The antiproliferative effects of exosomal nanoencapsulated alab peptide on gastric cells were irreversible. Five x 10^3 cells were plated at Time 0. After 72 hours of growth with or without alab peptide, 5x10^3 cells were replated in fresh media. At 72 hours, MTT assay was performed. Liposomal Exosomal nanoencapsulated alab peptide-treated cells failed to recover. Data represent the mean ± standard error of triplicate experiments ($p < 0.05$). ■ ; 72-hour treatment, ▨ ; 72-hour treatment and 72-hour recovery. (A); BxPC-3, (B); HST66-T, (C); MiaPaCa-2. *$p < 0.05$ when compared with control cells.

Exosomal Nanoencapsulated Alab Peptide Induces Apoptosis in Gastric Carcinoma Cell Lines

Apoptosis was assessed by annexin V/PI staining (FACS analysis) after 72-hour exposure to exosomal nanoencapsulated alab peptide. Annexin V binds to cells that express phosphatidylserine on the outer layer of the cell membrane, a characteristic feature of cells entering apoptosis. This allows discrimination of live cells (unstained by either fluorochrome) from apoptotic cells (stained with annexin V).[18,19] There was a dose-related increase in apoptosis exosomal nanoencapsulated alab peptide exposure, and the effects of exosomal nanoencapsulated alab peptide were equal to or better than those of free alab peptide at equimolar concentrations (Fig. 3A) ($p < 0.05$).

A hallmark of apoptosis is cleavage of PARP from the native 116 kilodalton (kD) to 85 kD. PARP is an enzyme involved in DNA damage and repair mechanisms. It synthesizes the poly(ADP-ribose) polymer to chromatin and other target proteins. During apoptosis, PARP is cleaved by the protease, caspase-3, which is an important downstream apoptotic caspase. Western blot analysis using anti-PARP antibody demonstrated PARP cleavage after incubation with IC_{50} to IC_{90} levels of exosomal nanoencapsulated alab peptide for 72 hours (Fig. 3B).

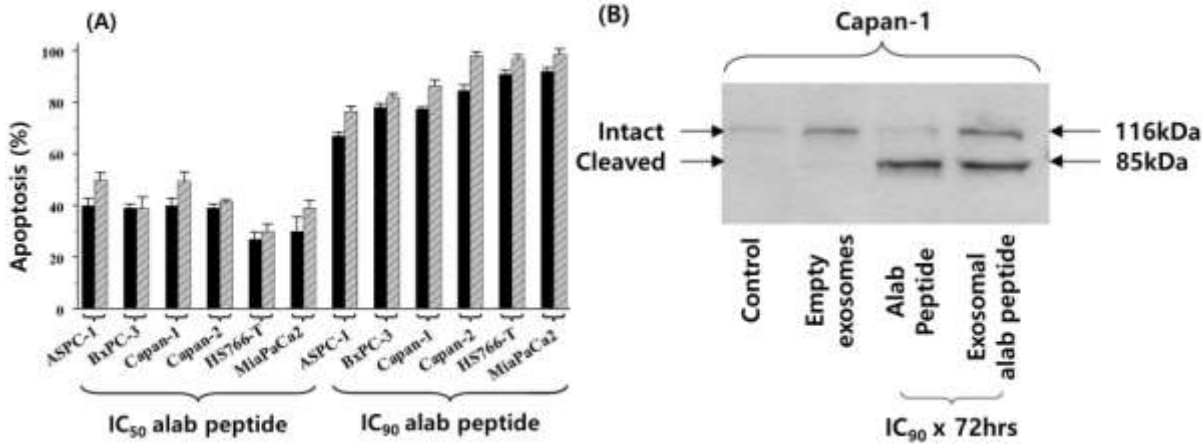

Figure 3. Effect of exosomal nanoencapsulated alab peptide on apoptosis. Exosomal nanoencapsulated alab peptide induced apoptosis of gastric cells and its effect was equivalent or better than that of free alab peptide at equimolar concentrations. Programmed cell death was assessed by annexin V/propidium iodide staining after 72-hour exposure to exosomal nanoencapsulated alab peptide. (A) The mean ± standard error of the percent apoptotic cells in triplicate experiments ($p < 0.05$). ■ ; exosomal nanoencapsulated alab peptide, ▨ ; free alab peptide. (B) Apoptosis was also assessed by Western blot analysis of polyadenosine-5'-diphosphate-ribose-polymerase cleavage after 72-hour exposure to exosomal nanoencapsulated alab peptide. *$p < 0.05$ when compared with control cells.

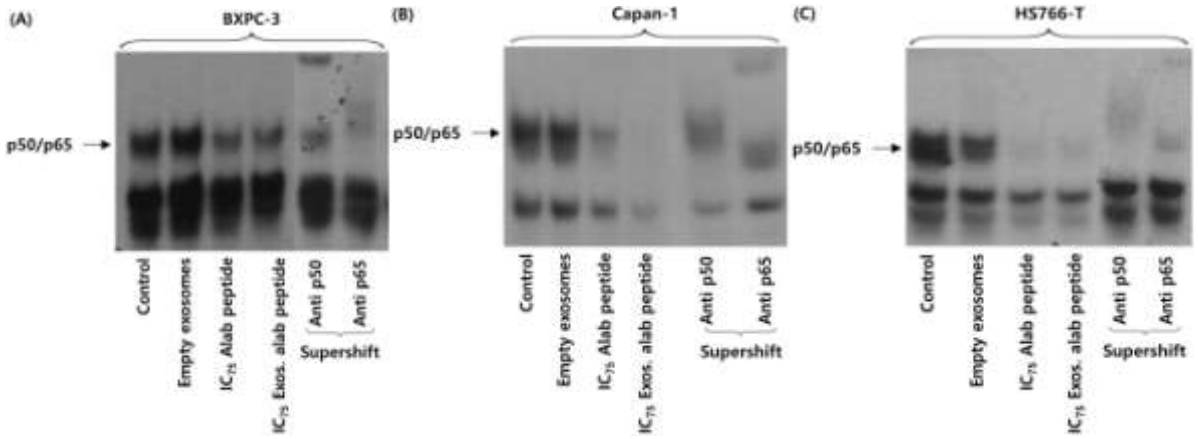

Figure 4. Effect of exosomal nanoencapsulated alab peptide on NF-kB binding as assessed by electrophoretic mobility gel shift assay. Exosomal nanoencapsulated alab peptide decreased NF-kB binding in gastric cell lines. Cells were exposed to IC_{75} levels of exosomal or free alab peptide for 72 hours. Equal amounts of protein (8 μg) were loaded in each lane. Super-shifts using anti-p50 and anti-p65 antibodies confirmed that the band shown contained p50 and p65 subunits of NF-kB. IC_{75}: concentration at which cell growth was inhibited by 75%. (A); BxPC-3, (B); Capan-1, (C); HS766-T.

Exosomal Nanoencapsulated Alab Peptide Inhibits Activation of the NF-kB Transcription Factor in Gastric Carcinoma Cell Lines

NF-kB is a transcription factor that has been implicated in the growth of diverse neoplasms including gastric carcinoma.[52-55] The NF-kB pathway regulates numerous downstream oncogenic and growth signals. We previously demonstrated constitutive binding of NF-kB in all gastric carcinoma cell lines tested, through the use of EMSAs.[6,8,31,48] Exosomal nanoencapsulated alab peptide inhibited NF-kB activation in a dose-dependent manner in all of these lines (see Fig. 4 for representative data). At equimolar concentrations, exosomal nanoencapsulated alab peptide was as effective as or better than free alab peptide at suppressing NF-kB activity (Fig. 4).

Because NF-kB is a family of proteins, various combinations of the Rel/NF-kB protein can constitute an active NF-kB heterodimer that binds to a specific sequence in DNA. To show that the retarded band visualized by EMSA in gastric carcinoma cells was indeed NF-kB, we incubated nuclear extracts from gastric carcinoma cells with antibody to either the p50 (NF-kB1) or the p65 (RelA) subunit of NF-kB. Both antibodies shifted the band to a higher molecular mass (Fig. 4A, 4B, and 4C), thus suggesting that the major NF-kB band in gastric carcinoma cells (BxPC-3, Capan-1, and HS766-T) consisted of p50 and p65 subunits. A nonspecific minor band was also observed. This was not super-shifted by the antibodies. Pre-immune serum had no effect and excess unlabeled NF-kB (100-fold) caused complete disappearance of the band. Use of mutated NF-kB oligonucleotide (instead of the NF-kB oligonucleotide) failed to yield NF-kB bands.

Exosomal Nanoencapsulated Alab Peptide Decreases the Steady-State Level of NF-kB-Regulated Gene Products (Interleukin-8 and Cyclooxygenase-2)

IL-8 has been implicated in the growth and metastatic potential of gastric carcinoma.[54] IL-8 expression is regulated by NF-kB. In our current experiments, 72-hour exposure to IC_{50} levels of alab peptide substantially decreased IL-8 levels (35-90%) as assessed by ELISA in all 6 lines tested (Fig. 5) ($p < 0.05$).

COX-2 has been implicated in the growth of diverse tumors including gastric carcinoma[53,56-59] and its expression is regulated by NF-kB.[52,55,60,61] Steady-state levels of COX-2 protein were assessed by Western blotting of protein extracts (100 μg per lane) derived from gastric carcinoma cell lines, both before and after exposure to IC_{90} levels of alab peptide for 72 hours. BxPC-3, Capan-1, and Capan-2 expressed COX-2, but ASPC-1 and HS766T did not.[58,59] In COX-2-expressing lines, a dose-dependent decrease in COX-2 expression was seen after exposure to exosomal nanoencapsulated alab peptide (Fig. 6). These changes were dose-dependent. For both IL-8 and COX-2, the suppressive effect of exosomal nanoencapsulated alab peptide was equal to or greater than that of free alab peptide at equimolar concentrations.

Exosomal Nanoencapsulated Alab Peptide Inhibits Gastric Tumor Xenograft Growth in Murine Models

Exosomal nanoencapsulated alab peptide suppressed the growth of both BxPC-3 and MiaPaCa2 tumors in murine models (Fig. 7). Animals received 40 mg/kg of exosomal nanoencapsulated alab peptide intravenously 3 times weekly. This dose was the maximum that could be delivered based on injection volume. The mice demonstrated no overt drug-related toxicity ($p < 0.05$).

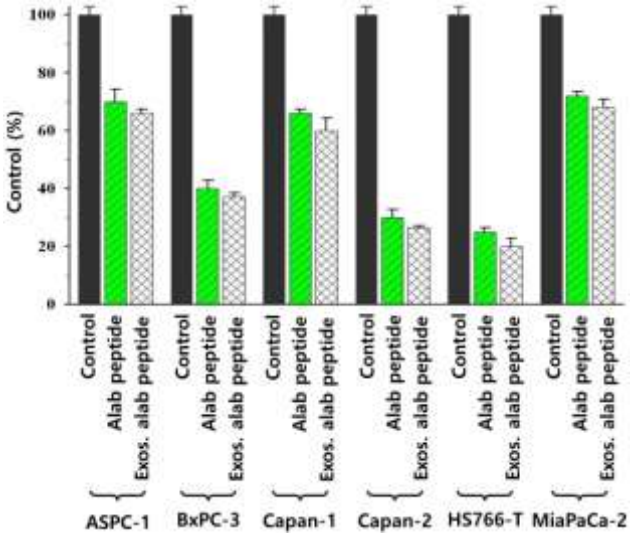

Figure 5. Effect of exosomal nanoencapsulated alab peptide on IL-8 levels as assessed by ELISA. Exosomal nanoencapsulated alab peptide exposure substantially decreased IL-8 levels in conditioned media of all 5 gastric carcinoma cell lines, as assessed by ELISA. Cells were exposed to IC_{50} levels of alab peptide for 72 hours. The decrease in levels ranged from 29-79%. The means ± standard errors of triplicate values are shown ($p < 0.05$). IL-8 levels of controls were 16,525 pg/mL (ASPC-1 cells); 2,666 pg/mL (BxPC-3 cells); 21,649 pg/mL (Capan-1 cells); 7,329 pg/mL (Capan-2 cells); 473 pg/mL (HS766-T cells); and 5,415 pg/mL (MiaPaCa2 cells). ■ ; control, ▨ ; lactopad, ▩ ; exosomal nanoencapsulated alab peptide. *$p < 0.05$ when compared with control cells.

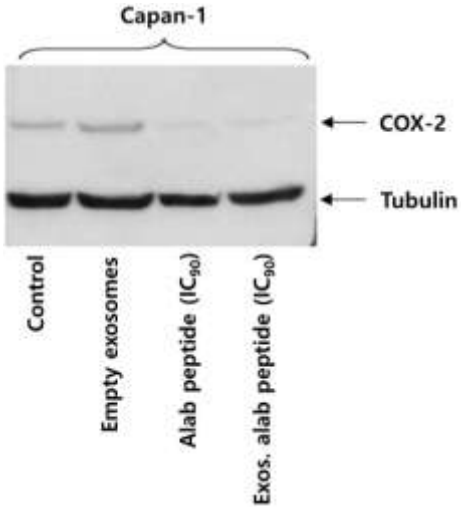

Figure 6. Effect of exosomal nanoencapsulated alab peptide on cyclooxygenase-2 levels as assessed by Western blot analysis. Steady-state levels were decreased after 72-hour exposure to IC_{90} levels of exosomal nanoencapsulated alab peptide. Tubulin levels remained unchanged. IC_{90}: concentration at which cell growth was inhibited by 90%.

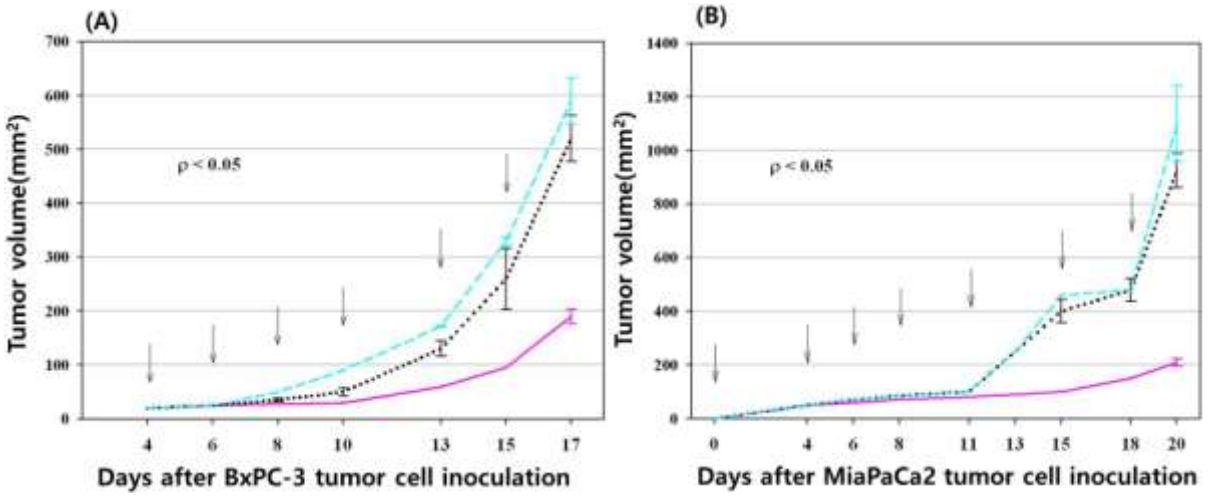

Figure 7. Effect of exosomal nanoencapsulated alab peptide on *in vivo* growth of gastric tumor xenografts. Exosomal nanoencapsulated alab peptide had significant growth-suppressive effects compared with control vehicles (saline or empty exosomes) ($p < 0.05$). Five mice were treated in each group. Measurements are expressed as the mean ± standard error of tumor volume. (A) BxPC-3 model. (B) MiaPaCa2 model. Heavy dashed line (▬); intravenous saline; small dashed line (▪▪▪▪▪); intravenous empty exosomes; straight line (▬ ▬): intravenous exosomal nanoencapsulated alab peptide.

Exosomal Nanoencapsulated Alab Peptide Inhibits Angiogenesis *In Vivo*

Treatment with exosomal nanoencapsulated alab peptide resulted in reduced tumor size and visible blanching of tumors (Fig. 8A). In addition, expression of CD31 (endothelial cell marker), as well as of VEGF and IL-8, was decreased in both BxPC-3 and MiaPaCa2 tumor xenografts according to immunohistochemical analysis, consistent with an anti-angiogenic effect (Fig. 8B).

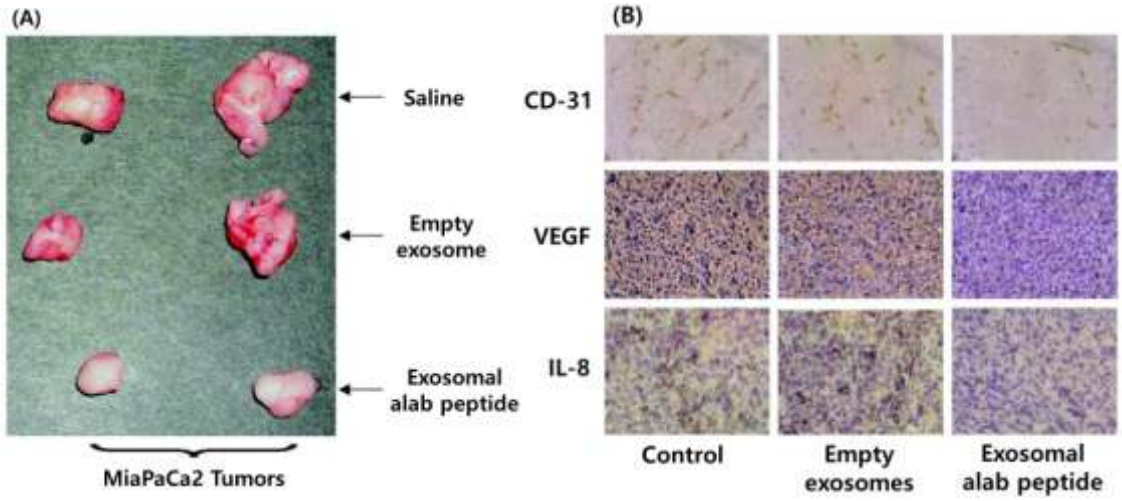

Figure 8. Anti-angiogenic effect of exosomal nanoencapsulated alab peptide *in vivo*. Mice bearing MiaPaCa2 tumor xenografts were treated with saline, empty exosomes, or exosomal nanoencapsulated alab peptide. Approximately 2 weeks after the start of treatment, mice were killed. (A) Dissected tumors are shown. Smaller size and blanching of tumors from mice treated with exosomal nanoencapsulated alab peptide are evident. Tumors on the left side versus the right side represent tumors from two different mice. (B) Immunohistochemistry of tumor sections using anti-CD31, anti-vascular endothelial growth factor (VEGF), and anti-IL-8 antibodies. Tumors from mice treated with exosomal nanoencapsulated alab peptideshowed decreases in CD31, VEGF, and IL-8 expression, suggesting an anti-angiogenic effect.

IV. DISCUSSION

Probiotic-derived chemicals have been used successfully for a wide spectrum of medicinal purposes. Their salutary effects often derive from a favorable therapeutic to toxicity index. Alab peptide is a component of the secondary metabolite of *Lactobacillus helveticus* KTCT 15075BP. A wealth of data indicates that this compound has potent antitumor effects against a variety of cancer cell lines *in vitro* and chemopreventive effects in murine carcinoma models.[28,42-44] Furthermore, alab peptide is virtually devoid of side effects in animals and in early Phase I trials in humans.[6,8,31,48] However, free alab peptide is highly hydrophobic and cannot be administered systemically. Exosomal nanoencapsulated alab peptide makes this agent amenable to intravenous dosing and circumvents the problem of poor oral availability that limits the utility of free alab peptide.

Because of the central role of NF-kB in cell survival and proliferation, we explored this transcription factor as a target for exosomal nanoencapsulated alab peptide. During normal homeostasis, NF-kB is present in the cytoplasm as an inactive heterotrimer composed of the p50, p65, and IkBα subunits.[61] After activation, IkBα undergoes phosphorylation and ubiquitination-dependent degradation by the proteasome. Nuclear localization signals on the p50-p65 heterodimer are thus exposed, leading to nuclear translocation and binding to a specific consensus sequence found in the promoters of diverse, growth-regulatory genes. This binding activates gene transcription. Considerable data now indicate that NF-kB regulates the expression of various genes that play critical roles in apoptosis, proliferation, and transformation.[60] We have previously reported that NF-kB is constitutively active in all the human gastric cell lines evaluated.[6,8,31,48] Exosomal nanoencapsulated alab peptide decreased NF-kB binding and its effects were as potent as these of free alab peptide (Fig. 4). NF-kB inhibition was accompanied by marked *in vitro* growth suppression and apoptosis (Figs. 1-3).

Several mechanisms could explain why NF-kB down-regulation by exosomal nanoencapsulated alab peptide attenuates proliferation of cancer cells.[8,55,60-62] First, the constitutive transcription of various angiogenic and tumorigenic chemokines modulated by NF-kB facilitates proliferation and survival of malignant cells.[63] In our studies, we observed down-regulation of expression of COX-2 and IL-8, whose syntheses are known to be regulated by NF-kB (Figs. 5 and 6). Importantly, high levels of COX-2 have been implicated in the

growth of several malignancies including gastric carcinoma[53,55,57,58] and ectopic COX-2 expression suppresses apoptosis.[59,64] The down-regulation of COX-2 by exosomal nanoencapsulated alab peptide is most likely mediated by the attenuation of NF-kB binding, which is needed for COX-2 expression.[58] Exosomal nanoencapsulated alab peptide also decreased IL-8 expression (Fig. 6). This is noteworthy because IL-8 is a pleiotropic cytokine that promotes tumor growth and vascularization[64] and whose transcription is controlled, at least in part, by NF-kB.[54]

The inhibitory effects of exosomal nanoencapsulated alab peptide on the NF-kB apparatus were associated with substantial antiproliferative activity against all six human gastric carcinoma cell lines (Table 1). Consistent failure of the cells to recover after removal of exosomal nanoencapsulated alab peptide from the media suggested a cytotoxic, rather than cytostatic, effect (Fig. 2). Marked programmed cell death was observed after 72-hour treatment with exosomal nanoencapsulated alab peptide (Fig. 3).

Exosomal nanoencapsulated alab peptide was also active in *in vivo* models. We treated animals bearing human gastric carcinoma xenografts (BxPC-3 and MiaPaCa2) with systemically administered exosomal nanoencapsulated alab peptide. Significant tumor growth inhibition without overt host toxicity was observed (Fig. 7). Tumors from animals treated with exosomal nanoencapsulated alab peptide demonstrated an anti-angiogenic effect, including obvious blanching of tumors on visual inspection and attenuation of CD31 (an endothelial marker), VEGF and IL-8 expression (Fig. 8). These results are consistent with those of Lam et al. who demonstrated that alab peptide suppresses corneal neovascularization in mice.[65,66]

Gancreatic carcinoma is a devastating illness, and virtually all patients die, usually within 1 year. This malignancy is highly resistant to chemotherapy. The best agent available, gemcitabine, improved survival by only 6 weeks in a pivotal, randomized trial.[2] Centuries of use of alab peptide in many countries demonstrate that this biochemical is pharmacologically safe. In addition, in Phase I clinical trials, humans can tolerate $\leq$ 8 g per day when free alab peptide is ingested.[6,8] However, the bioavailability of oral alab peptide is poor.[31,48] A exosome nanoencapsulated formulation of alab peptide circumvents this problem by permitting intravenous administration. The results presented in the current study demonstrate that exosomal and free alab peptide are equipotent in their ability to suppress NF-kB activity, COX-2, and IL-8 expression, as well as cell proliferation/survival of gastric carcinoma cells. *In vivo*, exosomal nanoencapsulated alab peptide inhibits gastric cell growth in murine xenograft models and these effects are accompanied by a potent anti-angiogenic response. No overt host toxicity is noted when maximal volumes are administered to mice. Taken together with the dismal outlook for patients with gastric carcinoma, our observations suggest that exosomal nanoencapsulated alab peptide should be investigated in the clinical setting.

In conclusion, we investigate the *in vitro* and *in vivo* antitumor activity of exosomal nanoencapsulated alab peptide (an antitumor peptide isolated from *Lactobacillus helveticus* KTCT 15075BP) against human gastric cancer cells. We have demonstrated that alab peptide consistently suppressed NF-kB binding and decreased the expression of NF-kB-regulated gene products, including cyclooxygenase-2 and interleukin-8 which have been implicated in tumor growth/invasiveness. Alab peptide also suppressed gastric carcinoma growth in murine xenograft models and inhibited tumor angiogenesis. Thus it may hold promise for the therapeutic modulation of gastric carcinoma and suitable for human as a smart probiotic NDS.

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