Effects of tachyplesin I on human U251 glioma stem cells

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Abstract. Glioblastoma, is one of the most malignant types of intracranial tumor with complex progressive cellular and underlying molecular events. The use of glioma stem cells (GSCs) offers a promising strategy for tumor therapy in the future. Tachyplesin I has been demonstrated to have potential anticancer activity and was first observed in leukocytes. In the present study, the GSC subset was isolated from U251 glioma cells and tachyplesin I was assessed for antitumor activity. As a result, the U251 cells exhibited certain GSC phenotypes, including the expression of stem cell biomarkers CD133 and nestin, when transferred into stem cell culture conditions. The GSCs were grown in an adherent manner in a medium containing serum, while the U251 glioma cells were suspended and cultured in serum-free medium. Tachyplesin I damaged the structure of GSC and inhibited the culture of GSC spheres in a time and dose-dependent manner. When tachyplesin I was administered at a concentration of 10-40 μg/ml, GSC differentiation was induced. GSCs treated with a low dose of tachyplesin I disrupted the plasma membrane and led to a loss of cytoplasmic organelles. These findings indicated that tachyplesin I had an effect on inhibiting tumor stem cells and demonstrated that tachyplesin I inhibited GSCs by disrupting the plasma membranes and inducing GSC differentiation.

Introduction

Tachyplesin I is a disulfide-stabilized β-hairpin antimicrobial peptide with 17 residues that can be isolated from hemocytes of the horseshoe crab (*Tachypleus tridentatus*). This peptide inhibits the growth of Gram-negative and Gram-positive bacteria at particularly low concentrations (1,2). A synthetic peptide from tachyplesin I has been demonstrated to decrease the growth of tumor cells *in vitro* and *in vivo* following linkage to the integrin homing arginine-glycine-aspartic acid (RGD) domain (3). Several studies had demonstrated that tachylesin inhibited the proliferation of tumor cells, including gastric adenocarcinoma, human hepatocarcinoma, prostate cancer and melanoma (3-5). In addition, tachyplesin I affects the differentiation of tumor cells (6). These findings suggest that tachyplesin I may be used to as an anti-tumor agent.

Malignant gliomas are aggressive brain tumors with limited therapeutic options. According to the type of glial cell in cancerous tissues, gliomas can be divided into four types, astrocytoma, oligodendroglioma or ependymoma (7). Glioma are composed of heterogeneous types of tumor cells that differ in their expression of markers and growth capacities (8). At present, glioma stem cells (GSCs) have been identified as the major reason for chemotherapy resistance in high-grade glioma (9). In addition, stem-like cells ($CD133^+$) have been found in malignant brain tumors and included cells expressing specific neural progenitor proteins, including nestin, SOX2 and OCT4 (10,11). These could initiate tumor formation following xenotransplantation (12).

The cancer stem cell (CSC) hypothesis provides new insight into the heterogeneity of types of malignant tumor (8). CSCs account for several important processes in carcinogensis, including tumor invasion, angiogenesis and recurrence, and tumor heterogeneity is determined, in part, by the presence of these CSCs (13,14). Therefore, the strategy in tumor therapy is aimed at destroying the tumorigenic CSCs (14,15). The present study aimed to examine the effects of tachplesin I on GSCs cells obtained from glioma U251 cell lines. It was hypothesized that tachplesin I is able to inhibit the proliferation of GSCs from glioma and is a potential antitumor drug.

Materials and methods

Tachyplesin I synthesis and cell culture. A peptide of tachyplesin containing the sequence NH_2-K-W-C-F-R-V-C-Y-R-G-I-C-Y-I-R-R-C-R-$CONH_2$ was synthesized by Hanyu Bioengineering Company (Shenzhen, China) with a purity of >95%. There were two disulfide bonds present in two positions, located between the 3rd and 16th cysteine and between the 7th and 12th cysteine.

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The NH_2-terminal was acetylated and the COOH-terminal was amidated to prevent its degradation. Prior to use, the peptides were dissolved in PBS at a concentration of 5 μg/μl.

U251 human glioma cells were purchased from the Chinese Academy of Sciences Cell Band (Shanghai, China), cultured in RPMI-1640 (Gibco-BRL Life Technologies, Grand Island, NY, USA) and supplemented with 10% fetal bovine serum (Gibco-BRL), 1% penicillin and streptomycin (Hyclone, Logan, UT, USA). The U251 cells were collected in the logarithmic growth phase. The U251 cells were thoroughly dissociated using 0.25% trypsin to prepare single-cell suspensions and then plated onto 10 cm dishes for culture in serum-free medium at a density of 30-50 cells/cm^2. Following incubation for 7 days at in 5% CO_2 and 100% humidity, clones of different morphological types were observed and adherent cells at the bottom were removed. Subsequently, the single-cell suspensions were cultured in 24-well plates at a clonal density and passaged at 1:2 or 1:3.

Self-renewal assay and induction of differentiation. The suspended cells (1x10^4 cells/ml) were cultured in neurobasal-A medium, which consisted of 50 ng/ml basic fibroblast growth factor (bFGF; Gibco-BRL), 50 ng/ml epidermal growth factor (EGF, Gibco-BRL) and 10 μl/ml B27 (Gibco-BRL) or RPMI-1640 supplemented with 10% fetal bovine serum (Gibco-BRL), 1% penicillin and streptomycin, and primary clone spheres were formed. Subsequently, primary clone spheres were digested using stem cell accutase (Gibco-BRL), centrifuged at 1,000 x g for 5 min and single cells were obtained for further culture to form secondary clone spheres.

Immunofluorescence microscopy and dyes. The second or third generation U251 tumor spheres were centrifuged and suspended in complete media. The cells were then dropped onto a polylysine embedding slice and fixed using 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 30 min. Following this, the cells were permeabilized with 0.3% Triton X-100 in phosphate-buffered saline (PBS) for 15 min and inhibited with 3% bovine serum albumin for 30 min before adding the following primary antibodies: Mouse anti-human Nestin (Abcam, Cambridge, MA, USA) and mouse anti-human CD133 (Abcam). The secondary goat anti-mouse immunoglobulin (Ig)G labeled with fluorescein isothiocyanate or cyanine-3 (Abcam) were added. Images were captured using an Olympus fluorescence microscope (Olympus, Toykyo, Japan). The contrast and brightness of the micrographs were then adjusted using Adobe Photoshop CS software (Adobe Inc., San Jose, CA, USA) for data presentation.

Administering of GSC with tachyplesin I and 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetraolium bromide (MTT) assay. The second or third generation stem cell spheres were administered with tachyplesin I. Following seeding for 24 h, the experimental groups were treated with reagent containing tachyplesin I in a concentration gradient (0, 10, 20, 40, 80 and 160 μg/ml). After 24 h, images were captured using an Olympus fluorescence microscope (Olympus).

Cell viability was determined using an MTT assay. Initially, U251 tumor spheres were digested to single cells using 0.25 % trypsin and seeded in a 96 well-plate. Tachyplesin I was used to treat cells when the concentration reached to 10^3-10^4 cells in each well. Following treatment with different concentrations of tachyplesin I for 24 or 48 h, the cultures were washed with PBS. Subsequently, MTT (0.5 mg/ml) was added to each well and the mixture was incubated at 37˚C for 4 h. Dimethyl sulfoxide (DMSO) of an equal volume was used instead of the culture medium to dissolve the formazan crysals. The mixture was then agitated at room temperature for 10 min and the absorbance of each well was determined at 490 nm using a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). All experiments were repeated six times and the data are expressed as the mean ± standard deviation (SD). The inhibitory rate of tyachyplesin was assessed using the following formula: inhibitory rate = (OD490 value of controls - OD490 value of tachyplesin I-treated U251 cells) / (OD490 value of controls - OD490 value of blanks) x 100%.

Transmission electron microscopy. The cells were fixed in 0.1 M phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde (Sigma-Aldrich) for 1 h. This was followed by fixation in 0.1 M phosphate buffer (pH 7.2) containing 1% OsO_4 (Sigma-Aldrich) for 1 h. The specimens were dehydrated in graded ethanol (Zhongshan Jinqiao, Beijing, China), embedded in epoxy resin (Electron Microscopy Sciences, Hatfield, PA, USA), cut into ultrathin sections and stained using uranyl acetate and lead citrate (Zhongshan Jinqiao). The stained ultrathin sections were observed by transmission electron microscopy (H-9500; Hitachi, Tokyo, Japan).

Scanning electron microscopy. For standard scanning electron microscopy, the cells were fixed in 0.1 M phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde for 1 h and subsequently fixed in 0.1 M phosphate buffer (pH 7.2) containing 1% OsO_4 for 1 h. The cells were then dehydrated in graded ethanol and critical-point air drying was performed, following treatment with isomyl acetate. The samples were sputter coated with OsO_4 and observed using a scanning electron microscope (S-4800; Hitachi, Tokyo, Japan) (17).

Statistical analysis. Data are expressed as the mean ± standard deviation. Differences between the mean values for individual groups were assessed using a Wilcoxon signed-rank test. Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Serum deprivation in U251 cells induces tumor stem cell sphere formation. Initially, the U251 cells were inoculated with serum-free medium. After 24 h, the U251 cells were adhesively grown with irregular protrusions. No proliferation of the U251 cells was observed (Fig. 1A). However, following culture in serum-free medium supplemented with B27, bFGF and EGF, the U251 tumor cells became non-adhesive and formed tumor spheres after 3 days (Fig. 1B). Subsequently, individual clonal spheres grew suspended in medium in the following 2-3 days. In addition, the majority of the clonal spheres demonstrated spherical or ovoid shapes and the cells exhibited a high proliferative activity. By contrast, the adhesive cells were programmed

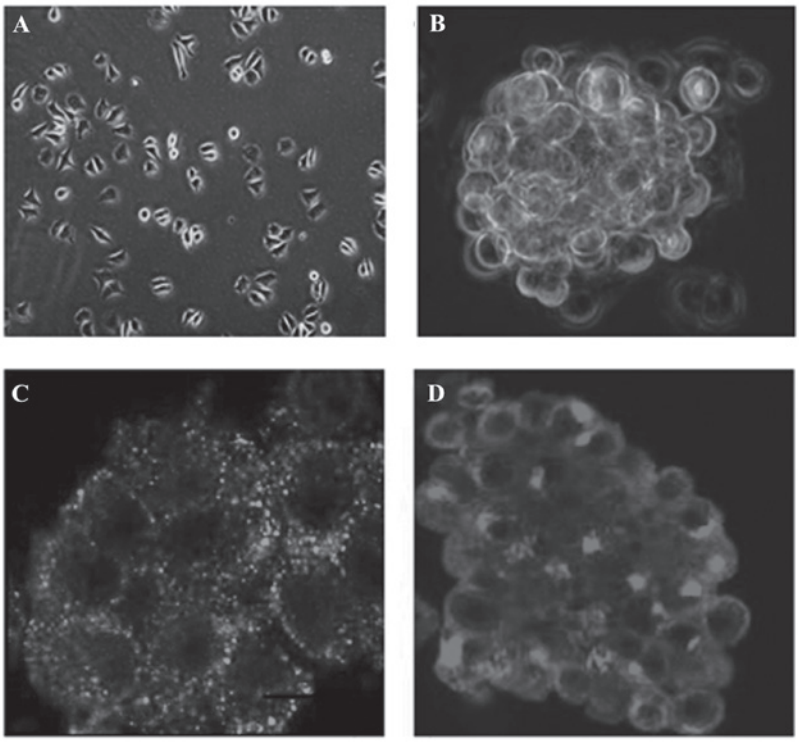

Figure 1. Differing growth patterns of U251 glioma cells cultured in different conditions. (A) U251 cells demonstrated adherent growth in serum-free medium for 24 h. (B) U251 cells demonstrated non-adherent sphere growth in serum-free medium. Immunofluorescenece analysis of the expression of (C) CD133 and (D) nestin. Magnification, x400.

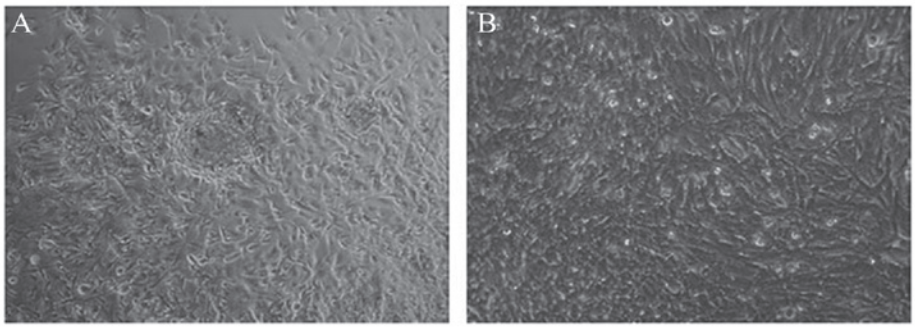

Figure 2. GSCs exhibit neruosphere-type, non-adherent growth when cultured in serum-supplemented media. (A) Morphology of GSC spheres cultured in serum-free media after 24 h. (B) After 72 h, adherent cells are fused, with spindle or stellate morphology. Magnification, x200. GSC, glioma stem cells.

towards cell death. CD133 and nestin, markers of brain CSCs, were detected using indirect immunofluorescence. As shown in Fig. 1C and D, CD133 and nestin were positively expressed in the suspended clone spheres. Based on these results, the suspended spheres derived from U251 cells formed glioma stem cells.

U251 stem cell growth patterns in different media. The U251 stem cells demonstrated different growth patterns in serum and serum-free medium. When isolated GSCs were cultured in medium with serum, after 4 h, the cell spheres grew against the wall of the flask. Following 12 h culture, the single cells had grown in the periphery of the stem cell spheres. As shown in Fig. 2A, the cells were rounded with some areas of cell flattening 24 h after culture. Adherent cells formed a monolayer at day 3 (Fig. 2B). No differences were observed in the morphological characteristics of GSCs following culture in serum medium compared with routine methods of U251 cell culture. However, when GSCs were cultured in serum-free medium, GSCs were observed to grow in a suspended manner with tumor stem-like morphology. In the following consecutive passages, conservation of GSC sphere morphology, characteristics and proliferative capacity was observed. Alterations in the growth pattern of the GSCs coincided with the medium used during culture. In the medium containing serum, the GSCs grew in an adherent manner, while the cells were suspended following culture in serum-free medium. Notably, the GSCs were plastic in response to their environment.

Inhibition of GSC proliferation by tachyplesin I is dose dependent. As GSCs are important in determining tumor progression, the present study hypothesized that the inhibition of GSCs contributed to limiting tumor proliferation. Therefore,

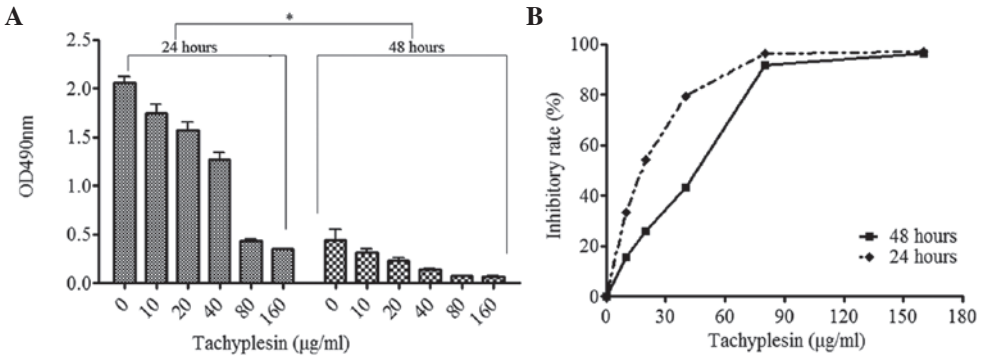

Figure 3. Growth inhibition of U251 GSCs following treatment for 24 or 48 h with varying concentration of tachyplesin. (A) 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetraolium bromide assay revealed that tachyplesin inhibited the GSC and U251 cells in a dose- and time-dependent manner (*P<0.05). (B) Inhibitory rate increased in accordance with the elevation in tachyplesin concentration. GSC, glioma stem cells; OD, optical density.

the present study examined the effects of tachyplesin I on the growth of GSCs. An MTT assay was used to evaluate the proliferation of GSC and U251 cells following treatment with various concentrations of tachyplesin I for 24 or 48 h. As shown in Fig. 3A, GSC proliferation was inhibited by tachyplesin I and the OD490 values were lower in the group treated for 48 h compared with those treated for 24 h (P=0.0313). Therefore, in addition to the decrease in OD490 with elevation in tachyplesin I concentration, the OD490 values declined between 24 and 48 h (Fig. 3A). These results indicated that the effects of tachyplesin I on GSC proliferation occurred in a dose and time-dependent manner. The growth inhibitory rates observed at the concentrations of 10, 20, 40, 80 and 160 μg/ml were 15.70, 25.82, 43.24, 91.70 and 96.45%, respectively, 24 h after treatment. After 48 h, the growth inhibitory rates were 33.47, 54.12, 79.44, 96.20 and 97.16%, respectively. These results demonstrated that tachyplesin I had marked anti-proliferative effects on the GSCs (Fig. 3B). In addition, it was observed that the higher the concentration of tachyplesin I, the stronger the anticancer-effects.

Tachyplesin I damages the structure of GSCs. Following treatment of the GSC spheres with a series of concentrations of tachyplesin I, observation using an inverted microscope after 24 h demonstrated destruction of the morphological characteristics.(Fig. 4). Furthermore, a notable feature of the brain tumor-initiating cells was the maintenance of tumor cells in an undifferentiated state. For this reason, new therapeutic approaches that force initiating cells to undergo differentiation to cease proliferation is being sought to improve cancer treatment (18). When tachyplesin I was administered at a concentration of 10-40 μg/ml, which induced GSC differentiation, certain cells extended outward from the spheroids in an adhesive manner (Fig 4B-D). As the concentration of tachyplesin I increased to 80 μg/ml (Fig 4E), there were several vesicles within the spheres and the cell contents leaked out. A 160 μg/ml concentration of tachyplesin I induced cell death (Fig. 4F).

Imaging of intracellular deposition of GSCs using transmission and scanning electron microscopy. To investigate the effects of tachyplesin I on GSCs, transmission and scanning electron microscopy were used to examine morphological changes. The primary cells were easily identified by their lack of a nucleolus and small quantities of cytoplasm and organelles (Fig. 5), consistent with stem cells (19). Initially, the GSCs had a clear cell structure (Figs. 5A and 6A) in the absence of tachyplesin I treatment. The nuclei were darkly stained and located in the central area with irregular morphologies. The cytoplasm contained abundant mitochondria, endosomes, Golgi apparati and endoplasmic reticula. The transmission electronmicrograph revealed the inner GSC structures, with the cells containing dendritic processes and folds. However, the cells treated with a low dose of tachyplesin I had a disrupted plasma membrane, which led to the loss of cytoplasmic organelles and a reduction in volume (Figs. 5B-D and 6B-D). Furthermore, a high dose of tachyplesin I promoted cell death (Figs. 5E-F and 6E-F).

Discussion

Several studies have revealed that the progress of carcinogenesis is driven and possibly accelerated by a subgroup of cancer stem-like cells with higher self-renewal capacity and the ability to differentiate (20). Despite the development of neurosurgery, chemotherapy and radiotherapy in previous decades, the mean survival rate of patients with malignant glioma is limited to 2 years. GSCs may be a potential target to improve the clinical efficacy of chemotherapy (20). In the present study, it was demonstrated that tachyplesin I inhibited tumor growth by inducing GSC differentiation and death.

The separation of stem-like cells from cancer cell lines offers a useful model for reflecting the biological activities present in the human body during carcinogenesis (22). valuable and accurate model of disease is possible by the establishment of tumor cell lines, which retain their stem cell properties to initiate cancer (22). In the present study, a large quantity of GSCs were obtained, which expressed CD133 and nestin, the typical stem cell biomarkers (23). These cells may represent the most malignant subset of tumor-initiating cells (9). The use of MTT assays revealed that tachyplesin I inhibited the proliferation of GSCs in a dose and time-dependent manner. However, due to a lack of sufficient evidence, the detailed mechanism of how tachplesin disrupts the progress of tumor stem-like cells remains to be elucidated. The cationic amino acids of tachyplesin I have been detected in negatively charged

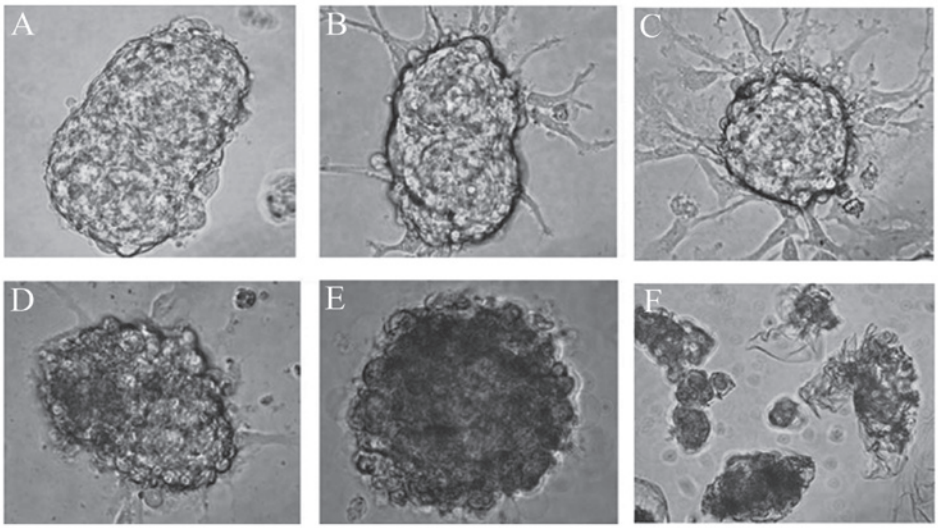

Figure 4. Morphological alterations following treatment with a series of concentrations of tachyplesin I. (A) 0, (B) 10, (C) 20, (D) 40, (E) 80 and (F) 160 μg/ml. Magnification, x400.

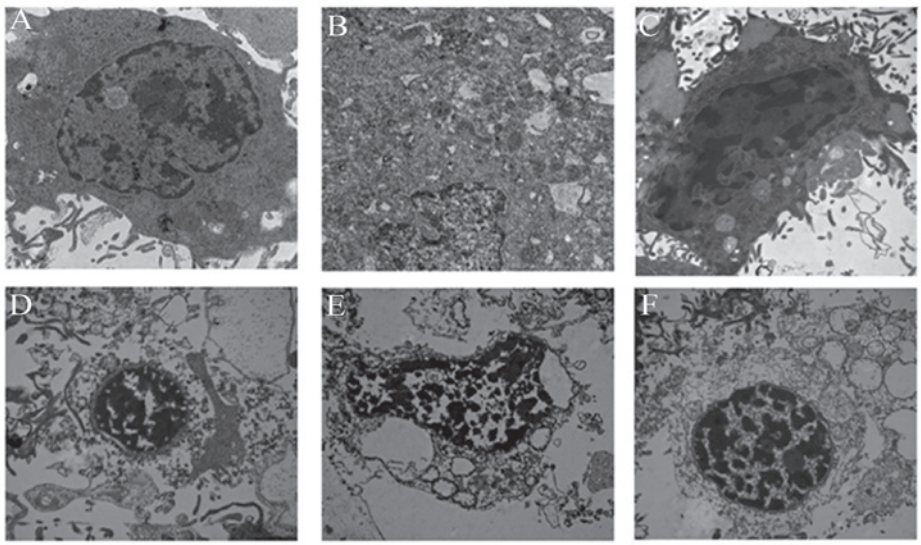

Figure 5. Imaging of glioma stem cell structural alterations following treatment with tachyplesin using transmission electron microscopy. (A) 0, (B) 10, (C) 20, (D) 40, (E) 80 and (F) 160 μg/ml tachyplesin I. Magnification, x20,000.

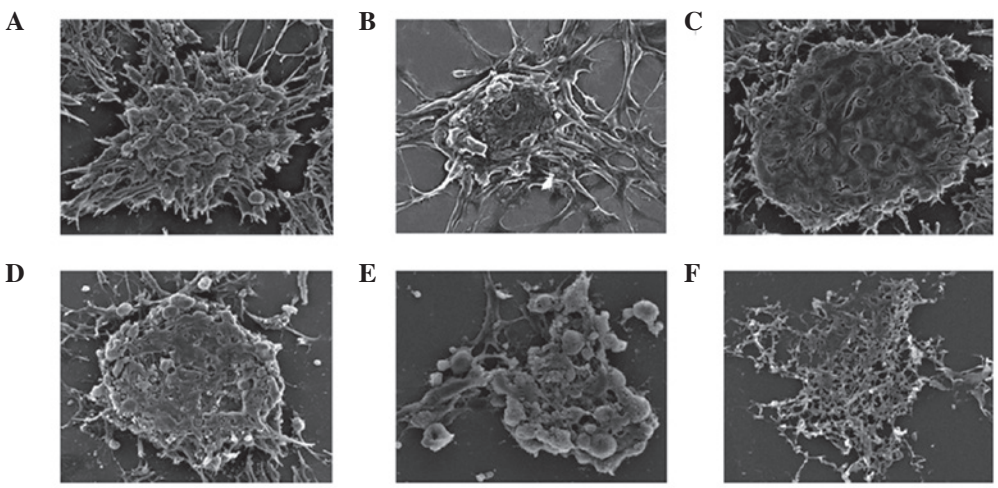

Figure 6. Imaging of glioma stem cells using scanning electron microscopy at different concentrations of tachyplesin I. (A) 0, (B) 10, (C) 20, (D) 40, (E) 80 and (F) 160 μg/ml tachyplesin I. Magnification, x500.

membranes, which interact with anionic head groups of phospholipids (24). In addition, tachyplesin I can destroy the integrity of the membrane by disrupting the lipid bilayer with their amphipathic helices (24). Above all, tachyplesin I is more likely to disrupt prokaryotic and mictochondrial membranes, as compared with the plasma membranes of eukaryotic cells due to the presence of zwitterion phospholipids in eukaryotic cells. The mechanism of the mitochondrial pathway, which induces tumor cell apoptosis is important in inhibiting tumor progression. In previous studies and in the present study, tachyplesin I was found to be important in the disruption of mitochondrial membranes and, therefore, it is proposed that tachyplesin I induces GSC apoptosis. Chen *et al* demonstrated that RGD-tachyplesin I led to upregulation in apoptosis associated with the mitochondrial and death receptor pathways. RGD-tachyplesin I activated the expression of either caspase 9, 8 and 3 or increased expression of the Fas ligand, and induced cell apoptosis. Furthermore, RGD-tachyplesin I also prevented tumor growth on the chorioallantoic membranes of chicken embryos and in syngeneic mice (3). These results highlight the potential use for tachyplesin I as an anti-tumor agent in clinical treatment in the future.

Tachyplesin I may have other roles in regulating GSC development. In the present study, tachyplesin I was found to induce GSC differentiation. Li *et al* demonstrated that thachyplesin downregulated the levels of mutant p52, cyclin D1 and CDK4 proteins and c-myc mRNA. It also induced the differentiation of human hepatocarcinoma cells (4) and induced the differentiation of the human hepatocarcinoma cell line, SMMC7721 (25). Furthermore, tachyplesin I not only inhibits the proliferation of tumor cells, but also regulates the cell cycle (4). In a previous study, tachyplesin I arrested the cell cycle at the G0/G1 stage (4) and induced an immune reaction. Chen *et al* (24) found that classic complement cascade reactions were induced following the linkage of tachyplesin I to hyaluronan in tumor cells or to C1q in the serum. This resulted in tachyplesin I disrupting the integrity of the tumor cell membrane and inducing cell death (24). Therefore, tachyplesin I has multiple effects in regulating tumor cell growth, including the induction of tumor cell apoptosis, differentiation and cell cycle arrest at the G0/G1 stage.

In conclusion, the present study demonstrated that tachyplesin I inhibited GSCs by disrupting the plasma membranes and inducing GSC differentiation. Further investigation of tachyplesin I, focussing on the detailed mechanisms of its role in inhibiting tumor stem cells is required. In addition, it is necessary to examine the role of tachyplesin I on glioma carcinogensis *in vivo*.

Acknowledgements

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