Perineural invasion (PNI) is a striking biological behavior observed in salivary adenoid cystic carcinoma (SACC). The present study was designed to establish a co-culture model of SACC cells with Schwann cells (SCs), and then study epithelial-mesenchymal transition (EMT) and the Schwann-like differentiation of SACC cells to investigate the likely molecular mechanism of PNI. The co-culture models of SCs with tumor cells (SACC-83, SACC-LM and MEC-1) were established using a Transwell system. An elevated concentration of brain-derived neurotrophic factor (BDNF) was detected by ELISA assay in the co-cultured medium of the SACC-83 group and SACC-LM group rather than the MEC-1 group. The EMT process and Schwann-like differentiation in SACC-83 cells were analyzed by RT-PCR, western blotting, immunofluorescence, photography, and migration and perineural invasion assays. The SACC-83 cells under the co-culture condition with SCs changed to a mesenchymal morphology and had higher migration and invasion capabilities compared with the solely cultured SACC-83 cells, accompanied by the downregulation of E-cadherin and upregulation of N-cadherin and vimentin. The co-cultured SACC-83 cells also developed Schwann-like differentiation with increased expression of SC markers, S100A4 and GFAP. However, inhibition of tropomyosin-related kinase B (TrkB) by K252a markedly blocked these effects. Additionally, the expression and correlation of TrkB, E-cadherin and S100A4 were analyzed by immunohistochemistry in 187 primary SACC cases. The levels of TrkB and S100A4 expression were both positively associated with PNI in the SACC cases, while E-cadherin expression was negatively associated with PNI. Elevated expression of TrkB was significantly correlated with the downregulated expression of E-cadherin and the upregulated expression of S100A4 in the SACC cases. Our results suggest that SCs play a pivotal role in the PNI process by inducing the EMT process and the Schwann-like differentiation of SACC cells via the BDNF/TrkB axis. Interruption of the interaction between SACC cells and SCs by targeting the BDNF/TrkB axis may be a potential strategy for anti-PNI therapy in SACC.

Introduction

Salivary adenoid cystic carcinoma (SACC) is one of the most virulent salivary gland cancers and accounts for ~30% of all salivary gland malignancies (1). Generally, SACC has a lengthy clinical course with potential local infiltration, hematogenous distant metastases, and poor response to classical chemotherapeutic approaches (2). A prominent hallmark of SACC is perineural invasion (PNI), which is the process of cancer cell invasion in, around and through the nerves. PNI of SACC is the key factor responsible for the incomplete surgical resection and the striking characteristic of SACC that distinguishes it from other salivary gland malignancies (3). The mechanisms involved in the PNI of SACC are still ambiguous although it has been investigated over a long period of time. Thus, it is crucial to establish a PNI model in vitro to mimic the perineural invasion process of SACC for further research on its molecular mechanisms.

Schwann cells (SCs) constitute the main cells of peripheral nerves, which are involved in the maintenance of axons and crucial for neuronal survival. When the peripheral nerve is injured or invaded by tumor cells, SCs exert essential function for maintaining the health of axons and the survival of neurons by producing a variety of neurotrophins (4). One of the most important neurotrophins is brain-derived neurotrophic...
factor (BDNF). BDNF acts on certain neurons of the central and the peripheral nervous systems, helping to maintain the survival of existing neurons and to encourage the growth and differentiation of new neurons and synapses (5). However, recent studies have revealed that BDNF and its receptor tropomyosin-related kinase B (TrkB) are also involved in the malignant progression of various tumors such as head and neck squamous cell carcinoma, hepatocellular carcinoma, and colorectal cancer (6,7). Our recent study for the first time found that BDNF and TrkB were highly expressed in SACC, and the elevated expression levels of BDNF and TrkB were significantly associated with PNI in SACC (8). Therefore, the molecular mechanisms of the BDNF/TrkB axis in the PNI of SACC require further study.

An increasing number of studies suggest that epithelial-mesenchymal transition (EMT), mediated by key transcription factors and induced by the local microenvironment, is a key biological process in epithelial tumor invasion and metastasis (9). During this process, tumor cells acquire increased migration and invasion abilities, and this provides a likely mechanism by which epithelial tumor cells leave primary sites and establish metastases. Recent research found that EMT is an important process involved in the PNI process in some neurotrophic cancers (8,10,11). In addition, accumulating evidence suggests that the expression of Schwann cell biomarkers is obviously increased in neurotrophic cancers (10,12). Some scholars hypothesized that Schwann-like cell differentiation might be also involved in the PNI process of neurotrophic cancers (13). Despite recognition of the PNI phenomenon in neurotrophic cancers, little progress has been made in the understanding of the molecular mechanisms of PNI in SACC. The present study was designed to investigate whether SCs could promote the process of EMT and the Schwann-like differentiation in the process of PNI in SACC cells via the BDNF/TrkB axis.

Materials and methods

Cell lines and cell culture. The human adenoid cystic carcinoma cell lines SACC-83 and SACC-LM were obtained from Peking University School of Stomatology (Beijing, China). The human mucopidermoid carcinoma cell line MEC-1 was kindly provided by the Department of Oral Biology, the Fourth Military Medical University (Xi’an, China). Tumor cells were maintained in RPMI-1640 medium with 10% fetal bovine serum (FBS) in a 5% CO₂ humidified atmosphere at 37°C. The primary culture and identification of SCs were carried out as in a previous study (14). SCs were isolated from the sciatic nerves of neonatal SD rats, which were obtained from the Laboratory Animal Center of the Fourth Military Medical University. The harvested cells, suspended in RPMI-1640 medium with 10% FBS, were plated onto dishes pre-coated with poly-L-lysine (PLL) and purified by means of a differential attachment technique.

Establishment of a co-culture system between tumor cells and SCs. A modified protocol of Transwell cultures was used, based on previous studies (15,16). A Transwell® (24 mm) with a 0.4-µm pore polyester membrane insert (Corning, Inc., Corning, NY, USA) was used to establish the co-culture system. SCs (1x10⁵/cm²) were seeded in the upper chamber, pre-coated with PLL, while 5x10⁴/cm² tumor cells were seeded in the lower chamber. Then the tumor cells were co-cultured with SCs in serum-free RPMI-1640 for 72 h. Solely cultured tumor cells or SCs were set as the negative controls.

Enzyme-linked immunosorbent assay (ELISA) analysis. ELISA assay was performed using the rat BDNF Quantikine™ ELISA kit (R&D Systems, Minneapolis, MN, USA). BDNF secretions from the medium of the solely cultured tumor cells, SCs and tumor cells co-cultured with SCs were measured after 72 h of culturing. The procedures recommended by the manufacturer were followed.

Photography and laser confocal imaging. After being maintained in serum-free RPMI-1640 medium for 72 h, SACC-83 cells in each group were photographed using a phase-contrast photomicroscope (Olympus, Center Valley, PA, USA). To visualize the cytoskeleton of the SACC-83 cells, cells were fixed in 4% paraformaldehyde, stained with phalloidin (Sigma-Aldrich, St. Louis, MO, USA) and counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen Inc., Carlsbad, CA, USA). Then the cytoskeleton of the SACC-83 cells was photographed by a Fluoview laser scanning confocal microscope (Olympus, Tokyo, Japan).

Quantitative RT-PCR analysis. Total RNA was isolated using Takara MiniBEST Universal RNA Extraction kit (Takara Bio, Inc., Otsu, Japan). Reverse transcription was completed by utilization of PrimeScript™ RT Master Mix (Takara Bio, Inc.). PCR amplification of the cDNA template was carried out using SYBR® Premix Ex Taq™ II (Takara Bio, Inc.) on CFX96™ Real-Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). β-actin functioned as the housekeeping gene. The relative expression level of the genes was calculated using the ΔΔCt method. Primers of the detected genes are listed in Table I.

Western blot analysis. Proteins extracted from each sample were separated on 8% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were blocked with non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 2 h at room temperature. Immunoblotting was performed using specific primary and secondary antibodies conjugated to horseradish peroxidase respectively. Primary rabbit polyclonal antibody for BDNF [Cell Signaling Technology Inc., (CST) Danvers, MA, USA, 1:1,000], TrkB (CST, 1:1,000), E-cadherin (CST, 1:1,000), N-cadherin (CST, 1:1,000), vimentin (CST, 1:1,000), S100A4 (CST, 1:1,000) and GFAP (CST, 1:1,000) were used. Bands were scanned using Chemidoc™ XR+ with Image Lab™ software (Bio-Rad Laboratories, Inc.) and quantification was carried out using Quantity One 4.4.0 software.

Scratch wound healing assay. The SACC-83 cells of each group were plated in the lower chamber of 24-well Transwell plates. When cells reached 80% confluency, the individual wells were wounded by scratching with a pipette tip and incubated with medium containing no FBS for 24 h. The cells were fixed in methanol and photographed to measure the wound distance.
Transwell perineural invasion assay. For the Transwell perineural invasion assays, 3x10^4 SACC-83 cells in 200 µl serum-free RPMI-1640 medium were seeded onto the Matrigel-covered inserts (for migration, 8 µm; Corning) in 24-well plates. The lower chamber was seeded with 5x10^4 SCs to simulate the perineural surrounding environment. The control groups consisted of seeded SACC-83 cells solely or treated with 100 nM K252a. After 24 h of incubation, no invaded tumor cells were removed, and the invaded cells were fixed in 95% ethanol and stained with methylrosanilinium chloride solution. Quantification was performed by counting the invaded cells in five independent fields under a magnification of x400.

Immunofluorescence staining. SACC-83 cells in each group were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. The samples were incubated with a primary rabbit polyclonal antibody for S100A4 (CST, 1:100) or GFAP (CST, 1:100) at 4˚C overnight, following by a secondary primary rabbit polyclonal antibody for S100A4 (CST, 1:100) or GFAP (CST, 1:100) at 4˚C overnight, following by a secondary antibody. Omitting the primary antibodies was set as a negative control.

Statistical analysis. All in vitro experiments were performed in triplicate. The t-test and the one-way ANOVA tests were performed to compare the results. The relationship between the expression of TrkB, E-cadherin, S100A4 and clinical PNI was performed by Spearman's rank correlation coefficient test. The correlation between the expression of TrkB and the expression of E-cadherin or S100A4 was evaluated by Spearman's rank correlation coefficient test. SPSS 17.0 software package (USA) was used to perform statistical analysis. P<0.05 was set the level of statistical significance.

Results

The expression of BDNF and TrkB in the interaction between SCs and tumor cells. The co-culture models vividly mimicked the crosstalk between tumor cells and SCs in the PNI process. ELISA analysis was carried out to detect the concentration of BDNF in the medium. BDNF was mainly produced by SCs and the concentration of BDNF in the medium of the co-cultured SACC cell lines with SCs was significantly higher than the sum of their solely cultured groups (P<0.05) (Fig. 1A). The concentration of BDNF in the medium of co-cultured MEC-1 cells with SCs exhibited no obvious changes (P>0.05).

The solely cultured or co-cultured tumor cells were collected and analyzed by quantitative RT-PCR and western blot assays. The expression of BDNF in the SACC cell lines exhibited no significant changes before and after co-culturing with the SCs (P>0.05), while the expression of TrkB was markedly elevated in the co-cultured groups compared with the solely cultured groups (P<0.05) (Fig. 1B and C). The expression levels of both BDNF and TrkB in the MEC-1 cell lines were low and exhibited no obvious changes before and after co-culturing with the SCs (P>0.05).

The solely cultured and co-cultured SCs were also tested by quantitative RT-PCR and western blot assays. The expression of BDNF in the SCs co-cultured with SACC-83 or SACC-LM...
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Figure 1. Expression levels of BDNF and TrkB were evaluated by ELISA, RT-PCR and western blot assays. (A) The concentrations of BDNF in the medium of solely cultured SCs and tumor cells as well as their co-cultured groups were detected by ELISA. The expression levels of BDNF in the medium of the co-culture groups of SACC cell lines with SCs were significantly increased but not in the MEC-1 cells. RT-PCR analysis (B) and western blot analysis (C) showed that the expression levels of TrkB in the co-culture groups of SACC cell lines were significantly upregulated while MEC-1 exhibited no similar changes. RT-PCR analysis (D) and western blot analysis (E) showed that SCs co-cultured with SACC cell lines expressed more BDNF than the solely cultured SCs and SCs co-cultured with MEC-1. The change in TrkB expression in SCs was not obvious in the solely culture groups and co-culture groups. *P<0.05; NS, not significant.

Figure 2. The expression levels of BDNF and TrkB in the SACC-83 cells were evaluated by RT-PCR and western blot assays. RT-PCR analysis (A) and western blot analysis (B) showed that the expression of TrkB in the co-cultured SACC-83 cells was significantly elevated compared with the solely cultured group while 100 nM K252a obviously blocked these effects. The expression of BDNF in each group did not change significantly. *P<0.05.

K252a interrupts the interaction between SCs and SACC-83 cells. To explore the effects of SCs on SACC cells, the co-culture model of SACC-83 cells with SCs was chosen for further in vitro studies. To explore the function of the BDNF/TrkB axis on SACC cells, TrkB inhibitor K252a (100 nM, Sigma-Aldrich) was added into the co-culture system. Quantitative RT-PCR and western blot analysis were used to investigate the expression of BDNF and TrkB in the solely cultured SACC-83 cells, co-cultured SACC-83 cells and co-cultured SACC-83 cells treated with K252a. The gene and protein expression of TrkB in the SACC-83 cells co-cultured with SCs was increased significantly compared with the solely cultured SCs (P<0.05), while the expression of BDNF in the SCs co-cultured with the MEC-1 cells exhibited no significant changes (P>0.05) (Fig. 1D and E). The expression of TrkB in the SCs exhibited no significantly changes before and after co-culturing with these tumor cells (P>0.05).
SCs promote the EMT progression of SACC cells via the BDNF/TrkB axis. Typical characteristics involved in the EMT process include cytoskeletal changes, increased motility and changes in a series of biomarkers such as E-cadherin, N-cadherin and vimentin. After co-culturing with SCs for 72 h, the SACC-83 cells were visualized using phase contrast microscopy and the cytoskeleton was photographed by laser scanning confocal microscope. The SACC-83 cells co-cultured with SCs exhibited obvious morphological changes compared with the solely cultured SACC-83 cells (Fig. 3A and B). The morphology of the SACC-83 cells co-cultured with SCs changed to a spindle-shape and a polygon-shape and the intercellular junction decreased. The ratio of spindle-shaped to polygon-shaped cells significantly decreased following treatment with K252a. Co-culturing with SCs significantly repressed the expression of E-cadherin (P<0.05), but promoted the expression of N-cadherin and vimentin in the SACC-83 cells (P<0.05) (Fig. 3C and D); and these effects were significantly blocked by K252a (P<0.05).

In addition, we investigated the effects of SCs on the motility of SACC-83 cells by a scratch wound healing and Transwell perineural invasion assays. Co-culturing with SCs significantly promoted the motility of the SACC-83 cells (P<0.05) (Fig. 3E and F). In contrast, inhibition of TrkB by K252a significantly impeded the motility of the SACC-83 cells even under the co-culture condition (P<0.05).

SCs promote the Schwann-like differentiation of SACC cells via the BDNF/TrkB axis. SACC cells co-cultured with SCs were assessed for expression of SC markers: S100A4 and GFAP. The gene and protein expression of S100A4 and GFAP in the co-cultured SACC-83 cells was markedly...
increased compared with the solely cultured SACC-83 cells (P<0.05) (Fig. 4A and B); these effects were significantly blocked by K252a (P<0.05). We also performed immunofluorescence staining to compare the changes in the expression of S100A4 and GFAP in the SACC-83 cells of each group. The number and fluorescence intensity of the S100A4- and GFAP-positive SACC-83 cells were significantly increased after co-culture with the SCs (P<0.05), while treatment with K252a significantly blocked this conversion (P<0.05) (Fig. 4C).
mainly expressed in the cytoplasm of the tumor cells, while E-cadherin was mainly expressed in the cell membrane and cytoplasm of the tumor cells (Fig. 5). TrkB and S100A4 were highly expressed in the SACC tissues, while they were only detected in some tuber cells and nervous tissues in the normal salivary glands. We also found that the staining intensity of TrkB and S100A4 around the peripheral nerve was obviously enhanced in the SACC specimens. Contrary to the expression of TrkB and S100A4, the expression of E-cadherin exhibited an opposite trend in the SACC and normal salivary gland. In the SACC tissues, the elevated expression levels of TrkB (92.0%, 172/187) and S100A4 (80.7%, 151/187) were significantly higher than the levels in the normal salivary gland tissues (15.0%, 3/20, P<0.01; 20.0%, 4/20, P<0.01, respectively). The expression of E-cadherin in the SACC tissues (47.6%, 89/187) was significantly lower than that in the normal salivary gland tissues (100%, 20/20, P<0.01).

Correlation between the expression of TrkB, E-cadherin and S100A4 and clinical PNI. As summarized in Table II, the expression levels of TrkB and S100A4 in the SACC tissues were both significantly associated with PNI (P<0.05), while E-cadherin was significantly inversely associated with PNI (P<0.05). Additionally, we assessed the correlation between the expression of TrkB and the expression of E-cadherin and S100A4 in the SACC specimens. As shown in Table III, the TrkB expression was significantly inversely associated with the E-cadherin expression (P<0.05) while significantly positively associated with S100A4 expression (P<0.05).

Discussion

PNI is a striking characteristic of SACC that is responsible for incomplete surgical resection, locoregional recurrence and distant metastasis (17). PNI also has been regarded as an independent indicator of aggressive behavior and poor prognosis in several neurotrophic cancers, most notably prostate and pancreatic cancer (18,19). The pathogenesis of PNI involves complex signaling between tumor cells and the nerves, and research in this area is still largely in its infancy. To investigate the likely mechanism of PNI in SACC, we hypothesized that the crosstalk between SACC cells and SCs in the PNI process plays a pivotal role. Thus, in this study, we established a co-culture model of SACC cells and SCs by a Transwell system to mimic the tumor-nerve cell interaction in the process of PNI.

BDNF, a member of the neurotrophin family, plays an important role in the maintenance of axons and survival of neurons when the peripheral nerve is injured (5). Yet in recent studies, increasing evidence has revealed that both BDNF and its receptor TrkB are overexpressed in a variety of malignances, including head and neck squamous cell carcinoma (21), breast cancer (6), colorectal cancer (7), hepatocellular cancer (22) and gastric cancer (23). Overexpression of these two markers in malignant tumors is consistently associated with a more aggressive behavior and poor prognosis (6,7,21-23). Furthermore, our previous studies demonstrated that overexpression of BDNF/TrkB is significantly correlated with clinical stage, perineural or vascular invasion, distant metastasis, and poor prognosis of SACC (8). In the present study, we mimicked the crosstalk between SACC cells and SCs in the PNI process plays a pivotal role. Thus, in this study, we established a co-culture model of SACC cells and SCs by a Transwell system to mimic the tumor-nerve cell interaction in the process of PNI.

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*aP<0.05 by Spearman’s rank correlation coefficient test.

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*aP<0.05 by Spearman’s rank correlation coefficient test.
data from the immunohistochemical staining also indicated that BDNF and TrkB were significantly overexpressed in the SACC specimens when compared with the normal salivary gland tissues. Interestingly, the staining intensity of BDNF/TrkB around the peripheral nerve in the SACC tissues was much stronger. These results suggest a potential role of the BDNF/TrkB axis in the PNI progression of SACC.

EMT is a process characterized by loss of cell polarity and intercellular adhesion molecules and acquisition of a fibroblast-like morphology with cytoskeleton reorganization (9). Increasing evidence suggests that EMT plays a crucial role in the acquisition of invasive and metastatic potential in a number of cancers, such as head and neck squamous cell carcinoma (21), breast cancer (24), pancreatic cancer (25) and lung cancer (26). Recent studies found that the BDNF/TrkB axis is involved in the EMT process in various cancers including SACC (8,21,27). Although the correlation between EMT and PNI has been rarely reported, it is plausible to infer that EMT plays an important role in the PNI process since it can confer tumor cells with increased migration and invasion abilities (8,10,11). In the present study, we hypothesized that SCs might promote the progression of PNI through the EMT process via the BDNF/TrkB axis.

Our data demonstrated that the phenotype of SACC-83 cells co-cultured with SCs changed from an epithelial morphology to a mesenchymal morphology accompanied by the conversion of EMT hallmarks (downregulation of E-cadherin and upregulation of N-cadherin and vimentin) and increased motility. This was in accord with the results from the immunohistochemistry of the SACC specimens that revealed that the expression of E-cadherin in SACC around the peripheral nerve was much lower than that in the normal salivary glands. Our Transwell PNI assay demonstrated that the co-cultured SCs significantly promoted the in vitro PNI ability of SACC-83 cells. In contrast, treatment with K252a markedly blocked this phenomenon in the SACC-83 cells. These results indicated that the interreaction of SCs and SACC-83 cells mediated the PNI process by inducing the EMT of SACC-83 cells via the BDNF/TrkB axis.

Cumulating evidence demonstrates that Schwann-like cell differentiation may be one of the likely PNI molecular mechanisms in neurotrophic cancers (12,13,28-30). Reed and Leonard firstly reported that the specific differentiation toward ‘neuroma-like’ qualities in melanoma may be relative to the PNI characteristics of melanoma cells (31). Additionally, Iwamoto et al demonstrated that perineural spread in desmoplastic melanomas was analogous to that of neurotropism in Schwann cells (32). Furthermore, Sun et al reported that myoepithelial cells differentiating into Schwann-like cells may be one of the mechanisms of PNI occurring in SACC (12). In the present study, we identified that the expression levels of SC markers S100A4, and GFAP were significantly upregulated in SACC cells when co-cultured with SCs, while inhibition of TrkB by K252a could not block the EMT process and Schwann-like differentiation induced by SCs completely. Thus we inferred that there must be other signaling pathways implicated in the interaction between SACC cells and SCs. Thus, the molecular mechanisms of PNI in SACC require further investigation.

We also analyzed the relationship between the expression of TrkB, E-cadherin, S100A4 and clinical PNI in the SACC specimens. We found that the elevated expression of TrkB and S100A4 and decreased expression of E-cadherin were significantly associated with the clinical PNI process. Moreover, the TrkB expression was significantly directly associated with the S100A4 expression and significantly negatively associated with the E-cadherin expression. Once more, these data confirmed that the BDNF/TrkB axis is implicated in the EMT process and Schwann-like cell differentiation in the development of PNI in SACC. However, in our in vitro cell co-culture experiments, we found that inhibition of the BDNF/TrkB axis by K252a could not block the EMT process and Schwann-like differentiation induced by SCs completely. Thus we inferred that there must be other signaling pathways implicated in the interaction between SACC cells and SCs. Thus, the molecular mechanisms of PNI in SACC require further investigation.

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References


