Abstract. Pathologically, >90% of bladder cancer is transitional cell carcinoma (TCC). Previously, brain-derived neurotrophic factor (BDNF) but not tropomyosin-related kinase B (TrkB) was found in normal urothelium. TrkB activation by BDNF has been shown to promote the progression of several cancers, however, the existence and functional roles of both BDNF and TrkB in TCC have not been elucidated. In this study, three human TCC cell lines, BFTC905, TSGH8301, and T24 were used for the investigation. Both BDNF and TrkB but not TrkA or TrkC identified by RT-PCR and Western blotting were found in these cell lines. Immunostaining demonstrated the cytosolic expression of BDNF and TrkB, as well as membranous expression of TrkB in these cells. BDNF released from three cell lines was also detected in culture medium by ELISA. The proliferation of BFTC905 cells was enhanced by recombinant human BDNF (rhBDNF) in vitro, which was associated with increased phospho-TrkB and phospho-ERK levels. In contrast, TrkB-Fc chimeric protein served as BDNF scavenger eliciting cytotoxicity. Addition of rhBDNF in these cell lines cultured in poly-HEMA [Poly(2-hydroxyethyl methacrylate)] coated dishes for 48 h did not confer resistance to anoikis. Increased phospho-Akt expression was observed transiently within an hour after rhBDNF administration but disappeared 24 h later. Weekly injections of 100 ng rhBDNF into the cancer cell-loading site for 6 weeks promoted BFTC905 xenograft growth in SCID mice. Daily injection of 5 μg TrkB-Fc chimeric protein into the tumor 2 weeks after tumor cell implantation delayed tumor growth concomitant with phospho-TrkB suppression in xenografts. These results indicate that BDNF binding to TrkB receptor is a survival signal for TCC cells. Drugs that block BDNF or TrkB may provide a new and potential approach for TCC therapy.

Introduction

Bladder cancer is one of the most commonly diagnosed malignancies in the world (1). Pathologically, >90% of bladder cancer is transitional cell carcinoma (TCC). Current therapies for bladder cancer include surgery, radiation, and/or chemotherapy (2). However, the median survival time is approximately one year once distant metastasis occurs, despite aggressive treatment (3). Research should therefore direct toward new strategies for bladder cancer treatment.

BDNF (brain-derived neurotrophic factor) is a member of the neurotrophin family. BDNF exerts its effects by binding to the tyrosine kinase receptor TrkB (tropomyosin-related kinase B), which regulates neuronal survival and differentiation (4). TrkB has also been found in many normal non-neuronal tissues, such as macrophages, and epidermis (5). BDNF but not TrkB has also been found in normal urothelium, but the function of BDNF in urothelium remains unclear (5,6). In cancer biology, BDNF and/or TrkB have also been found in several malignant tissues, e.g., neuroblastoma (7), pancreatic ductal adenocarcinoma (8), prostate cancer (9), lung cancer (10), and hepatocellular carcinoma (HCC) (11). TrkB has also been demonstrated to mediate resistance to anoikis, apoptosis resulting from the loss of cell-matrix interaction (12). This phenomenon may result from the activation of the PI3K-AKT pathway in ovarian cancer cells (13). The neurotrophin receptor family TrkA and TrkC also mediate the progression of some tumors (14-17), but their expressions in normal urothelium and TCC have not been reported. Results from this study provide the first evidence that BDNF and TrkB are survival factors in TCC, indicating that these targets may be exploited for clinical therapy.

Materials and methods

Correspondence to: Dr Ted H. Chiu, Institute of Pharmacology and Toxicology, Tzu Chi University, Chung Yang Rd., Hualien 970, Taiwan, R.O.C.
E-mail: thchiu@mail.tcu.edu.tw

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from ATCC, with the grading of 3 and 2, and 2/3, respectively. They were provided by Dr Jui-I Chao (Department of Biological Science and Technology, National Chiao Tung University, Hsin-Chu, Taiwan), and the cells were maintained as described previously (20). For anoikis study, 24-well dishes were pre-coated with 5 or 10 mg/ml poly-HEMA (poly-2-hydroxyethyl methacrylate), Sigma, St. Louis, MO), and the cells will survive under suspension culture conditions if they exhibit the ability of anoikis resistance.

**Cell growth and MTT cytotoxicity assay.** Growth of 8x10^5 BFTC905 cells were plated in 10 cm dish containing RPMI-medium supplemented with 1% fetal bovine serum (FBS) plus 0-20 nM rhBDNF (recombinant human BDNF, Calbiochem, Merck KGaA, Darmstadt, Germany) (day 0). Subsequently, the cells were re-cultured with fresh medium plus rhBDNF on days 2 and 4. Cell counts were calculated by a hemocytometer on days 2, 4, and 6. For anoikis study, 1x10^4 TCC cells were cultured in poly-HEMA coated dishes with 1% or 10% FBS plus 0-50 nM rhBDNF for 48 h. Subsequently, cells were collected and stained with trypan blue (Sigma), and only live cells were counted in the hemocytometer.

For cytotoxic effect, 1x10^5 BFTC905 cells per well were seeded in 96-well plates. Recombinant human TrkB-Fc chimeric protein (R&D Systems, Minneapolis, MN) at 0-20 μg/ml was added to the medium containing 1% FBS for 72 h. Human immunoglobulin (IgG, Sigma) treatment was served as a negative control. Conventional MTT (methylthiazolyldiphenyl-tetrazolium bromide, Sigma) assay was followed (20).

**Mouse xenograft model.** The NOD.CB17-Prkdc^scid/Tcu (SCID) male mice, 3-4 weeks of age, were provided and cared by the Laboratory Animal Center of Tzu Chi University. Preliminary data showed that, among the 3 cell line examined, only BFTC905 cells grew successfully and consistently in these mice, and hence only BFTC905 xenograft was studied. One million BFTC905 cells in 500 μl PBS (phosphate-buffered saline) was implanted s.c. into the right inguinal area of the mice (day 0). Starting on day 0, 100 ng rhBDNF in 50 μl distilled water or distilled water (control) were injected s.c. weekly for 6 weeks into the loading site of cancer cells or directly into the tumor when it became palpable. Tumor dimensions were measured weekly and the tumor volumes were calculated using the formula: [1/2]xa x b^2, where a and b represent the larger and smaller tumor diameters, respectively. Mice were sacrificed and the xenografts were removed after one 6-week treatment, and liver/inguinal/peritoneum were also checked for possible metastases.

In another study, 5 μg of recombinant human TrkB-Fc chimeric protein or human IgG dissolved separately in 50 μl PBS, or 50 μl normal saline, were administered s.c. daily similar to those of rhBDNF. The first day of drug injection was started on day 14 after tumor cells implantation, and the mice were sacrificed on day 35.

**Immunocytochemistry (ICC).** Cancer cells were fixed on the gelatin-coated slides with methanol (Riedel-deHaen, Sigma-Aldrich, Seelze, Germany) at -20°C for 5 min, and then dried in room air. For membranous staining only, cancer cells on gelatin-coated slides were fixed with 4% paraformaldehyde (Riedel-deHaen) at 37°C for 2 h. Further steps of immunostaining used by Department of Pathology, Buddhist Tzu Chi General Hospital were followed (21). Primary antibodies of anti-BDNF (sc-546, Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-TrkB (sc-8316, Santa Cruz), anti-TrkA (AF175, R&D Systems), and anti-TrkC (AF373, R&D Systems) were used. Exclusion of primary antibody was used as a negative control.

**Enzyme-linked immunosorbent assay (ELISA).** Human BDNF Quantikine™ and human phospho-TrkB DuoSet™ ELISA

**Table I. Primer sequences of BDNF, TrkA, TrkB, TrkC, and GAPDH.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>BDNF</td>
<td>Forward 5'-ATGACCATCTTTTTCCCTTACTAGT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CTATCTTCCCCTTTTTAATGGTCA-3'</td>
</tr>
<tr>
<td>BDNF</td>
<td>Forward 5'-GGGTTTATCTTTTGAGAATTTTG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CTTTGCTTATCCCTACCCACT-3'</td>
</tr>
<tr>
<td>TrkB</td>
<td>Forward 5'-TGTCGCCACGTCTCTGCAAATTCAG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GCCCTTGGAGGACTCTGTACCC-3'</td>
</tr>
<tr>
<td>TrkB</td>
<td>Forward 5'-TGACACCAAGGTATTAATCGACAG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-ACGGTAAATAGTCCGTCGTAGAT-3'</td>
</tr>
<tr>
<td>TrkA</td>
<td>Forward 5'-CGGAAACTGCGCGGTGGCGGTCGAG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TTTCAAGGCTATAAATAATATT-3'</td>
</tr>
<tr>
<td>TrkC</td>
<td>Forward 5'-CGCCCGGAGGATGGGAAACCTCTTC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GTCTCTGGCAAGGCTGGCTG-3'</td>
</tr>
<tr>
<td>TrkC</td>
<td>Forward 5'-AATTCAACTCTCTTGCCTCTCT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TGATGTGCAGGACACTTTGAGT-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward 5'-CGGAGGCAACGATTGGCTGTATAT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-AGCCCTTCTCATGTTGGAGACG-3'</td>
</tr>
</tbody>
</table>

These sequences are derived from the mRNA of human BDNF transcript variant 1, Genbank accession no. NM_170735; TrkB transcript variant a, NM_006180; TrkA transcript variant 1, NM_001012331; TrkC transcript variant 1, NM_001012338; and GAPDH, NM_002046.
kit purchased from R&D Systems were used. BDNF secretion was measured from 5x10^7 cells incubated in 10 ml medium plus 10% FBS for 24 h. After centrifugation, the supernatants were filtered through Amicon Ultra centrifugal filter devices (10K & 50K Da cut off, Millipore, Billerica, MA) to remove interfering proteins. For phospho-TrkB detection, BFTC905 cells (1x10^7) were treated with PBS or 20 nM rhBDNF for 0-30 min or after initial overnight treatment with 0.5 μg/ml of TrkB Ab or IgG followed by 20 nM rhBDNF for 30 min. Phospho-TrkB levels in xenograft were measured 2 h after the last daily injection of a 21-day TrkB-Fc chimeric protein treatment. The procedures recommended by the manufacturer were followed.

Reverse transcription-polymerase chain reaction (RT-PCR). BDNF1 and BDNF2 primers from coding and non-coding region, respectively (Protec Technology, Taipei, Taiwan), and similarly TrkA, TrkB and TrkC primers were designed to check the mRNA levels using RT-PCR. GAPDH (glycer-aldehyde-3-phosphate dehydrogenase) mRNA was used as an internal control. The primer sequences are shown in Table I. RT-PCR steps as used previously were followed (22). A total of 39 PCR cycles under sequential settings of 95˚C for 1 min, 57˚C for 1 min and 72˚C for 1 min was used.

Western blot analysis. Standard procedures were followed (20). Primary antibodies used included anti-BDNF, anti-TrkB, anti-TrkA, anti-TrkC, phospho-ERK (extracellular related kinase, #9106, Cell Signaling, Danvers, MA), and phospho-AKT (#4051, Cell Signaling). Expression of ERK2 (sc-154, Santa Cruz), AKT (sc-8312, Santa Cruz), or actin (sc-1616, Santa Cruz) was used as the internal standard. Phosphorylated protein signals were augmented by Can Get Signal™ immuno-stain (Toyobo, Osaka, Japan). The intensity of immunoreactive proteins was scanned and the intensity calculated by NIH software ImageJ V.1.40.

Statistical analysis. Data are expressed as mean ± SEM (standard error of mean) and evaluated by Student’s t-test, one-way or two-way ANOVA based on individual data. In all cases, p<0.05 was considered statistically significant and labeled by an asterisk on the figures.

**Results**

**BDNF and TrkB were expressed in TCC cells.** Nucleotide sequences from database reveal that BDNF, TrkB, TrkA and TrkC possess large untranslated sequences. Thus, 2 sets of primers, one from the coding region and the other from the 3' untranslated region, were designed to estimate the mRNA levels using RT-PCR. Both coding and non-coding regions of BDNF and TrkB but not TrkA and TrkC mRNA fragments were expressed in 3 TCC cell lines (Fig. 1). Both pro-BDNF and BDNF protein were expressed in these cell lines (Fig. 2A). Only TrkB (both full-length and truncated, 145 and 95 kDa, respectively) but not TrkA or TrkC immunoreactive proteins were found in these cell lines (Fig. 2B-D). TrkB (95 and 145 kDa) proteins were also found in BFTC905 xenograft tissue (Fig. 2B).

Immunocytochemical analysis of three cell lines (Fig. 3) demonstrated BDNF protein expression in the cytoplasm of cells under methanol fixation, which disrupted cell membrane, allowing primary antibody to penetrate into the cytoplasm. However, DAB stained cells were not observed if bladder cancer cells were fixed with paraformaldehyde, which maintained an intact plasma membrane. These results confirmed the cytoplasmic localization of BDNF in human bladder cancer cells. No DAB stains were found when primary antibody was eliminated from the reaction mixture (control panel). In addition to the presence in the cytoplasm, BDNF was detected in culture medium by ELISA assay. No BDNF was detected in the culture medium containing 10% FBS without cells. Furthermore, TrkB protein expression was found in the cytosol plus cytosol/plasma membrane under methanol fixation but was observed only on the plasma membrane under paraformaldehyde fixation (Fig. 3). This demonstrated...
that TrkB existed both in cytosol and on plasma membrane of human bladder cancer cells. Results revealed neither TrkA nor TrkC expression in these cell lines.

BDNF promoted BFTC905 cell survival but did not resist anoikis, while recombinant human TrkB-Fc chimeric protein induced cytotoxicity in vitro. Consistent xenograft tumor formation was observed only after BFTC905 cell implantation, thus BFTC905 cells were chosen for further in vitro studies. Fig. 4A shows that 20 nM rhBDNF significantly enhanced BFTC905 cell proliferation with the medium containing 1% FBS (p=0.02, two-way ANOVA). ELISA showed that phosphorylated TrkB protein was significantly elevated 10 min after the addition of 20 nM rhBDNF and persisted for at least 30 min (Fig. 4B, p=0.02, n=4). The rhBDNF-induced phospho-TrkB expression was blocked when cells were pre-treated overnight with 0.5 μg/ml TrkB Ab (Fig. 4B). ERK activation is one of the signaling pathways of TrkB activation (23), and it mediates TrkB-induced cancer progression (24). Importantly, ERK activation predicts poor outcome in TCC patients (25). Thus, we examined the dynamic expression of phospho-ERK after rhBDNF stimulation in BFTC905 cells. Increased expression of phospho-ERK was also observed after 20 nM rhBDNF administration (Fig. 4C).

The effect of a BDNF scavenger, recombinant human TrkB-Fc chimeric protein, was investigated to substantiate the functional interaction between the endogenous BDNF and TrkB. TrkB-Fc chimeric protein elicited significant cytotoxicity under serum-deprived conditions with the medium containing 1% FBS (Fig. 4D). Cultured after 48 h in both 5 and 10 mg/ml poly-HEMA coated dishes, all TCC cells in the presence of 0-50 nM rhBDNF showed nuclear trypan blue staining (data not shown). Consequently these TCC cell lines did not exhibit the ability to resist anoikis despite exogenous BDNF administration. The time-course of the expression of phospho-AKT after 50 nM rhBDNF administration in BFTC905 cells maintained in poly-HEMA coated dishes showed increased expression after 1 h but disappeared 24 h later (Fig. 4E).

**Discussion**

The present study demonstrated the existence of BDNF and TrkB in three TCC cell lines. The exogenous rhBDNF promoted bladder cancer growth in vitro and in vivo. We have also shown that rhBDNF activated the phosphorylation of TrkB and its downstream signaling molecule ERK in vitro. The expression of phospho-TrkB protein was blocked by TrkB-Fc chimeric protein. The growth and the level of phospho-TrkB in xenograft tumors were suppressed by TrkB-Fc treatment.

As expected, TrkB receptor is a plasma membrane protein, but cytoplasmic expression has been also observed in normal...
cells (26,27), ovarian cancer cells (13), as well as TCC cells in our study. However, the function of cytosolic TrkB requires further investigation. Although TrkB, TrkA or TrkC has been found to mediate certain tumor progression (14-17), our results indicate that neither mRNA nor protein expression of TrkA or TrkC can be detected in TCC. These data demonstrate the absence and the lack of functional roles of TrkA and TrkC in bladder cancer cells, further strengthening the importance of BDNF and TrkB in bladder cancer growth. Importantly, previous studies reported that TrkB has not been found in normal urothelium (5), so TrkB blockade might be a specific target for TCC with no physiological influence on normal urothelium.

Evaluation of the cellular growth cultured in poly-HEMA coated dishes is an established method to observe the cellular death by anoikis (28). Anoikis resistance mediates the survival
of cancer cells detached from extracellular matrix with multiple signaling pathways including BDNF-TrkB axis, and further promotes metastasis (29). Although both BDNF and TrkB were expressed in BFTC905, T24, and TSGH8301 cells, these TCC cells did not resist anoikis in the polyHEMA dishes. Besides, exogenous rhBDNF did not improve the survival in this suspension culture condition despite transient AKT activation. These results indicated that BDNF and TrkB do not rescue these 3 TCC cell lines from anoikis in such culture system. Only one previous study mentioned the ability of anoikis resistance in TCC cells through COX-2 (cyclooxygenase 2) transfection into human bladder cancer cell line EJ, also under poly-HEMA culture conditions (30). Maybe poly-HEMA coated dish is not suitable for anoikis resistance study in TCC cells, and other HUANG et al: BDNF, TrkB AND TRANSITIONAL CELL CARCINOMA

Figure 4. Effects of exogenous rhBDNF on BFTC905 cells in vitro. (A) BFTC905 cells cultured in undernourished medium (1% FBS) were treated with 10 or 20 nM rhBDNF. Cell proliferation during 6-day incubation was obtained. *p<0.05. (B) Time course of exogenous 20 nM rhBDNF stimulation on the expression of phospho-TrkB was obtained by ELISA. Pre-treatment with 0.5 μg/ml of TrkB Ab for 24 h blocked the stimulating effect of 20 nM BDNF. *p<0.05 compared to the absence of BDNF. (C) Time course of phospho-ERK expression after 20 nM rhBDNF activation. (D) Cytotoxic effect of TrkB-Fc was measured by MTT assay 72 h after treatment. Cell viability of IgG treatment was set as 100%. *p<0.05 vs IgG treatment. (E) Time course of phospho-AKT expression after 50 nM rhBDNF activation cultured in poly-HEMA dishes.

Figure 5. Growth of BFTC905 xenografts in vivo. (A) 100 ng rhBDNF in 50 μl distilled water was injected subcutaneously into the loading site of cancer cells once a week, starting the day following cancer cell injection. A total of five rhBDNF administrations was given. The tumor volumes were calculated using the formula: \[ \text{Volume} = \frac{1}{2}ab^2 \], where a and b represent the larger and smaller tumor diameters, respectively. (B) Recombinant human TrkB-Fc chimeric protein at 5 μg/50 μl PBS was administered s.c. daily into the loading site of cancer cells or directly into the tumor starting on day 14 after implantation. Similar administration of 50 μl normal saline (N/S) or human IgG at 5 μg/50 μl PBS served as the control. (C) Expression of phospho-TrkB in xenograft after 21-day TrkB-Fc treatment was measured by ELISA.
methodology such as culture dish with ultra-low attachment coated polystyrene surface may be used for further investigation (31).

Among the three cell lines tested, only BFTC905 cells successfully grew in SCID mice. Subcutaneous xenografts for T24 (32) and TSGH8301 (33) in SCID mice have been reported. However, these studies chose higher tumor cell loading (4x10^6 and 1x10^7, respectively) compared to our study (1.0x10^6), so consequently tumor masses might grow more quickly and easily. The characteristics of microenvironment on invasiveness for three cell lines might differ, resulting in different speeds of mass formation (32). Besides, the IgG leakage in SCID mice might be another problem (34). The percentage of IgG leakage in SCID mice might be different between our study and the previous reports. In the present study, xenografts of BFTC905 cells consistently grew under condition of less tumor cell loading compared to previous studies. This result indicates that BFTC905 cell line might be a better candidate for in vivo TCC study.

Exogenous BDNF promoted TCC cell growth only under serum-deprived medium containing 1% FBS, but without proliferative effect in 10% FBS medium (unpublished data). Similarly, Pearse et al (35) demonstrated that BDNF promoted the survival of multiple myeloma cells without stroma. Yang et al (11) also showed that BDNF significantly enhanced the proliferative properties of HCC cells. We anticipated that the effects of exogenous BDNF would emerge from a more stressful environment, which was demonstrated in the medium containing 1% FBS. Detectable BDNF in culture media confirmed its autocrine existence in TCC cells. Furthermore, phosphorylation of TrkB was observed in the absence of exogenous rhBDNF, and the cytotoxicity appeared after scavenging BDNF with TrkB-Fc established the autocrine function of BDNF. In addition to its autocrine origin, circulating BDNF elaborated by certain cells or tissues may also exist. Thus, in vivo BDNF administration into mice may reflect enhanced paracrine and autocrine effects of BDNF. We also found that the effects of exogenous BDNF would make a more stressful environment, which was demonstrated in the medium containing 1% FBS.

The recombinant human TrkB-Fc chimeric protein is composed of the extracellular domain of human TrkB fused to the C-terminal histidine tagged Fc region of human IgG1, allowing it to specifically bind human BDNF. TrkB-Fc sequesters BDNF and consequently functions as a specific TrkB inhibitor (37-39). Blockade of BDNF/TrkB interaction by TrkB-Fc delayed the progression of the xenograft bladder tumor in SCID mice through inhibition of TrkB phosphorylation. We also found that pan-Trk inhibitor K252a also suppressed BFTC905 xenograft tumor growth (unpublished data). Therefore, the blocking of TrkB receptor may become a new strategy for bladder cancer therapy.

Recent reports have demonstrated the important roles of BDNF/TrkB in TCC cell lines. Exogenous BDNF promoted TCC xenograft growth, and BDNF ablation elicited opposite effects. These results indicate that BDNF and TrkB receptor play important roles in regulating TCC growth. Targeting the BDNF or TrkB receptor may provide an important new approach for treating TCC.

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