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Original Article

Brain-derived neurotrophic factor plus vascular endothelial growth factor additively promotes early growth of the transitional cell carcinoma cell line BFTC905 *in vitro* and *in vivo*

Pei-Chun Lai [a], Ying-Chin Yang [b], Chuan-Chu Cheng [c], Ted H. Chiu [d], Yen-Ta Huang [d,e,f,*]

[a] *Department of Pediatrics, Buddhist Tzu Chi General Hospital, Hualien, Taiwan*
[b] *Division of General Surgery, Buddhist Tzu Chi General Hospital, Hualien, Taiwan*
[c] *Department of Medical Research, Buddhist Tzu Chi General Hospital, Hualien, Taiwan*
[d] *Department of Pharmacology, Tzu Chi University, Hualien, Taiwan*
[e] *Department of Medicine, Tzu Chi University, Hualien, Taiwan*
[f] *Surgical Intensive Care Unit, Buddhist Tzu Chi General Hospital, Hualien, Taiwan*

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ABSTRACT

Objective: The attempt to block one type of receptor tyrosine kinase (RTK) signaling has been considered a main strategy for cancer therapy; however, cancer cells may survive using alternative RTK signaling. Thus, targeting multiple RTKs simultaneously may be a better treatment strategy. Previously, we demonstrated that brain-derived neurotrophic factor (BDNF) via activating tropomyosin receptor kinase B (TrkB) is a survival factor for transitional cell carcinoma (TCC). Others have reported that autocrine vascular endothelial growth factor (VEGF) pathways also play a crucial role in TCC growth. We aim to examine the *in vitro* and *in vivo* effects of BDNF plus VEGF on the TCC cell line BFTC905 derived from the bladder cancer of a Taiwanese patient with black foot disease.

Materials and Methods: Cell numbers were counted after administration of 50 nM BDNF and/or VEGF to the BFTC905 or primary human urothelial cell (HUC) cultures for 96 hours. The volumes of the xenograft tumors were measured 6 weeks after weekly injections of 100 ng BDNF and/or VEGF into the cancer cell-loading site of NOD.CB17-Prkdc^{scid}/Tcu mice. The expression of TrkB in HUCs and VEGF receptor FLT-1 in BFTC905 cells and HUCs, as well as CD34 and Ki-67 in the xenograft tumors was determined by Western blotting.

Results: The effects of increasing BFTC905 cell numbers were as follows: BDNF + VEGF > BDNF > VEGF > control after 96 hours of treatment. The VEGF receptor FLT-1 was expressed in BFTC905 cells. Exogenous BDNF and/or VEGF did not promote the proliferation of HUCs. In addition, TrkB and FLT-1 were very weakly expressed in HUCs. After treatment for 5 weeks, the effects of exogenously administered BDNF and/or VEGF on the xenograft tumor volume were as follows: BDNF + VEGF > BDNF > VEGF > control. However, similar tumor sizes with few metastases were found in the four groups of mice after treatment for 6 weeks. The expression levels of the angiogenic marker CD34 and proliferative marker Ki-67 were also similar among these four groups of xenograft tumors.

Conclusion: Exogenous BDNF plus VEGF additively promotes the early growth of BFTC905 *in vitro* and *in vivo* without affecting the normal urothelium. Targeted blockade of both TrkB and VEGF signaling might be an effective treatment for adjuvant bladder cancer therapy or in the early stage of high-grade TCC.

1. Introduction

Urinary bladder cancer is the ninth most common malignancy in the world [1]. Pathologically, more than 90% of bladder cancers are transitional cell carcinoma (TCC) [2,3]. Once metastasis involves distal organs, the median survival is approximately 1 year despite

* Corresponding author. Surgical Intensive Care Unit, Buddhist Tzu Chi General Hospital, 707, Section 3, Chung-Yang Road, Hualien, Taiwan. Tel.: +886 3 8565301x2064; fax: +886 3 8561465.
E-mail address: uncleda.huang@gmail.com (Y.-T. Huang).

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aggressive treatment [4]. Thus, new treatment for metastatic TCC should be explored.

The products of tyrosine kinase genes regulate cellular functions, including proliferation, differentiation, and motility [5]. Several receptor tyrosine kinases (RTKs) overexpressed in cancer tissues have been confirmed to be oncogenes [6]. Overexpression of some growth factors and their corresponding RTKs reported in bladder cancer, such as epidermal growth factor receptor and platelet-derived growth factor receptor β, has been found to be associated with poor clinical outcomes [7]. However, there have been only a few clinical trials of target therapy for bladder cancer because some preclinical trials for certain RTKs showed disappointing results [7]. Unfortunately, resistance from multiple mechanisms has been reported in target therapy using one drug to block a specific RTK [6]. In addition, results from microarray and high-density oligonucleotide array showed that cancer cells can survive through several RTK signal pathways [8,9]. Therefore, the strategy for future cancer therapy is to find a drug that blocks multiple targets or combinations of drugs to block multiple RTKs [10,11].

In our previous reports, brain-derived neurotrophic factor (BDNF) and tropomyosin receptor kinase B (TrkB) were found in three TCC cell lines (BFTC905, TSGH8301, and T24) [12]. In human TCC specimens, increased immunostaining for BDNF and TrkB was observed compared with that in normal tissues [13]. The proliferation and invasiveness of BFTC905 cells were enhanced by BDNF [12]. TrkB antibody suppressed proliferation, elicited cytotoxicity, and inhibited migration [14]. These results indicate that both BDNF and TrkB play important roles in the progression of TCC. Vascular endothelial growth factor (VEGF) receptors were overexpressed in TCCs and bladder tumor specimens [15,16]. The autocrine VEGF pathway was also involved in the survival and proliferation of TCCs [15]. In this study, we demonstrated that BDNF plus VEGF additively promoted the early growth of the Taiwan TCC cell line BFTC905 *in vitro* and *in vivo*. In the future, targeted blockade of both VEGF receptor (VEGFR) and TrkB signaling may be an effective adjuvant postoperative bladder cancer therapy or treatment in the early stage of high- grade TCC when patients refuse surgery.

2. Materials and methods

2.1. Cell line and cell culture

TCC cell line BFTC905 with a pathologic grade of three was purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). BFTC905 is derived from a patient in Taiwan with black foot disease [16]. These cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, L-glutamine (0.03%), and sodium bicarbonate (2.2%). Primary human urothelial cells (HUCs) were also used in this investigation (ScienCell Research Laboratories, Carlsbad, CA, USA), and maintained in a special culture medium (No. 4312, ScienCell Research Laboratories). Both BFTC905 cells and HUCs were cultured in 5% CO_2 at 37°C in a humidified incubator.

2.2. Cell proliferation assay

The growth of 1×10^4 BFTC905 cells or 5×10^3 HUCs was determined in a 10-cm dish containing RPMI medium supplemented with 1% FBS or a special medium, respectively, plus 50 nM recombinant human BDNF (PeproTech, Rocky Hill, NJ, USA), or 50 nM recombinant human VEGF (PeproTech) or both. Administration of an equal volume of phosphate buffered saline (PBS) was used as a control. Subsequently, the cells were recultured with BDNF, VEGF, or both on hour 48. Cell counts were calculated using a hemocytometer 48 hours and 96 hours after incubation.

2.3. Mouse xenograft model

NOD.CB17-Prkdc^{scid}/Tcu male mice, 3∼4 weeks old, weighing approximately 20 g and raised by the Laboratory Animal Center of Tzu Chi University were chosen for the study. Mice were housed in isolation cages with autoclaved bedding in a specific pathogen-free room with 12-hour light-dark cycles. Sterile water and food were supplied by the staff of the Laboratory Animal Center of Tzu Chi University.

1.0×10^6 BFTC905 cells suspended in 500 μL PBS were implanted subcutaneously into the right inguinal area of the mice. Tumor dimensions were measured weekly and the tumor volumes were calculated using the formula: $[1/2] \times a \times b^2$, where a and b represent the largest and smallest tumor diameters, respectively [17]. Body weight was also measured weekly. One hundred ng of recombinant human BDNF, VEGF, or both in 50 μL distilled water was injected subcutaneously into the loading site of cancer cells weekly, starting the day following tumor injection. Drug treatment lasted for 5 weeks. In the control group, 50 μL distilled water was administered into the same location. After 6 weeks, mice were sacrificed using a carbon dioxide asphyxiator, and a necropsy was performed immediately. The livers and lungs were also checked for possible metastases.

2.4. Western blot analysis

Cells were homogenized in ice-cold cell extract buffer, pH 7.6, containing 0.5 mM dithiothreitol, 0.2 mM EDTA, 20 mM hydroxyethyl piperazineethanesulfonic acid (HEPES), 2.5 mM $MgCl_2$, 75 mM NaCl, 0.1 mM Na_3VO_4, 50 mM NaF, and 0.1% Triton X-100. Protease inhibitors, including 1 μg/mL aprotinin, 0.5 μg/mL leupeptin, and 100 μg/mL 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), were added to the cell suspension. Xenograft tumors were homogenized with the same buffer solution as the cell culture except 1% Triton X-100 was used, followed by the addition of a complete protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, IN, USA). The protein concentrations were determined by a protein assay kit (Bio-Rad, Hercules, CA, USA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed as previously described [12]. All antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), including anti-TrkB (sc-8316), anti-VEGF receptor FLT-1 (sc-31173), anti-Ki-67 (sc-15402), and anti-CD34 (sc-9095). Expression of α-tubulin (anti-α-tubulin, sc-8305) or ERK2 (anti-ERK2, sc-154) was used as the internal standard. The intensity of immunoreactive proteins was calculated by National Institutes of Health software ImageJ V.1.40. The intensity ratio was calculated by dividing the intensity of Ki-67 or CD34 by that of α-tubulin.

2.5. Statistical analysis

Data were expressed as mean ± standard error of mean (SEM) and evaluated by one-way analysis of variance (ANOVA) to compare the *in vitro* and *in vivo* growth difference at each time point among the four groups of animals (i.e., controls, and treatment groups with BDNF, VEGF, and BDNF + VEGF). Posttests with Bonferroni multiple comparison test after repeated measures one-way ANOVA were used. In all tests, $p < 0.05$ was considered statistically significant and labeled by an asterisk on the figures. No significant change was labeled "n.s." (not significant).

3. Results

3.1. BDNF plus VEGF exerted the largest effect on the increase of BFTC905 cell numbers in vitro

As shown in Fig. 1A, administration of 50 nM BDNF, 50 nM VEGF or both for 48 hours did not increase the numbers of BFTC905 cells compared with the controls ($n = 3$; $p = 0.27$, one-way ANOVA). However, treatment for 96 hours significantly increased their numbers ($n = 3$; $p < 0.0001$, one-way ANOVA). The order of the effect at 96 hours following drug administration was BDNF + VEGF > BDNF > VEGF > controls. However, the difference in cell counts between BDNF and VEGF treatment, and between control and VEGF treatment, did not reach statistical significance. The number of cells in the presence of BDNF plus VEGF was $(1.75 \pm 0.38) \times 10^5$, which was approximately the number of cells combined under BDNF [$(9.23 \pm 0.5) \times 10^4$] and VEGF [$(7.70 \pm 0.27) \times 10^4$] treatment. Expression of TrkB was confirmed in our previous report [12], and FLT-1, a VEGFR, was also expressed in BFTC905 cells (Fig. 1B).

3.2. BDNF and/or VEGF had no effect on the proliferation of HUC cells in vitro

Growth factors that promote the proliferation of TCC cells without affecting the proliferation of normal urothelium would be considered the best candidates for therapy in targeted blockade. A slower proliferation rate was observed for HUC cells (from 5×10^3 to 1.5×10^4) compared with the rapid proliferation of BFTC905 cells in control medium (from 1×10^4 to 6.14×10^5) in 96 hours. The HUC cell counts among control, BDNF, VEGF, and BDNF plus VEGF treated groups were not statistically different until 96 hours after

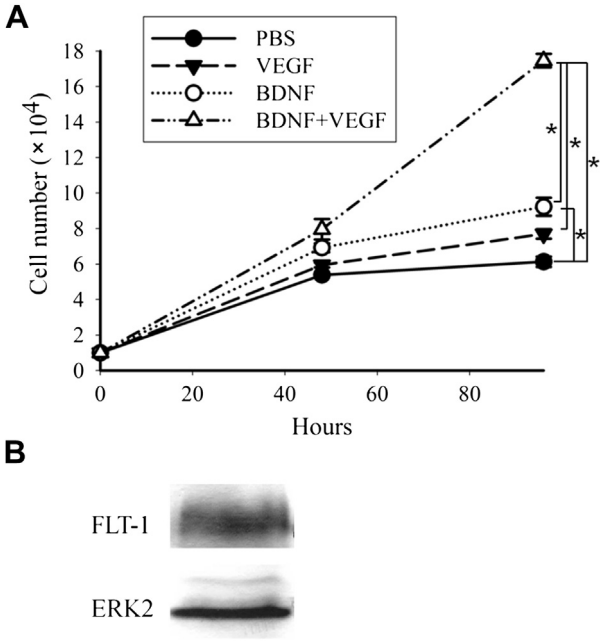

Fig. 1. (A) Effects of exogenous brain-derived neurotrophic factor (BDNF) and/or vascular endothelial growth factor (VEGF) on BFTC905 cells *in vitro*. BFTC905 cells cultured in undernourished medium (1% fetal bovine serum) were treated with 50 nM BDNF and/or VEGF at 0 and 48 hours. Cell proliferation was measured during a 96-hour incubation. Values are mean ± standard error of the mean from three separate experiments. * $p < 0.05$. (B) Expression of FLT-1 in BFTC905 cells detected by Western blotting. ERK2 = extracellular regulated kinase; FLT-1 = vascular endothelial growth factor receptor 1.

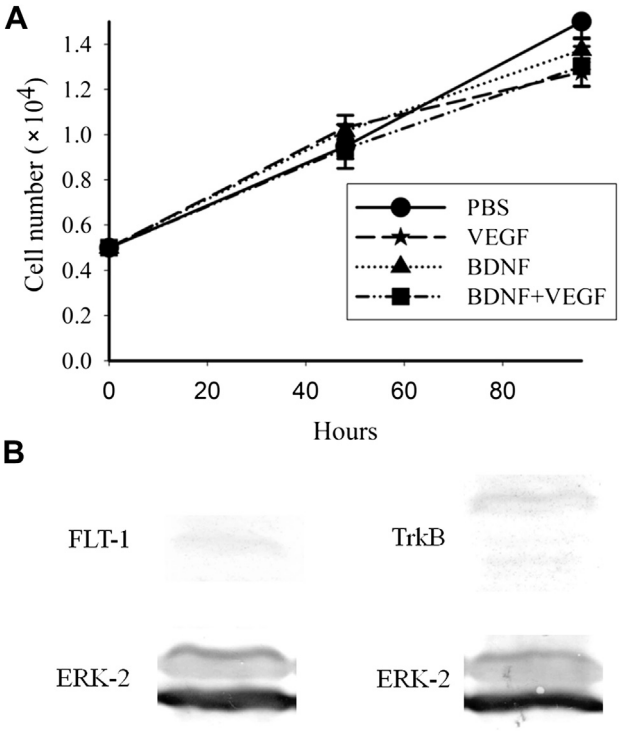

Fig. 2. Effects of exogenous brain-derived neurotrophic factor (BDNF) and/or vascular endothelial growth factor (VEGF) on human urothelial cells (HUCs) *in vitro*. (A) HUCs were treated with 50 nM BDNF and/or VEGF at 0 and 48 hours. Cell proliferation was measured during a 96-hour incubation. Values are mean ± standard error of the mean from three separate experiments. (B) Very weak expression of FLT-1 and TrkB in HUCs is detected on Western blotting. ERK2 = extracellular regulated kinase; FLT-1 = vascular endothelial growth factor receptor 1; TrkB = tropomyosin receptor kinase B.

administration (Fig. 2A). Furthermore, we found that TrkB and FLT-1 were very weakly expressed in HUC cells on Western blotting (Fig. 2B).

3.3. BDNF plus VEGF exhibited the largest growth effect on BFTC905 xenograft tumors in vivo following 35 days of treatment

BDNF, VEGF, and BDNF plus VEGF administered weekly at the tumor loading sites in the mice all promoted xenograft tumor

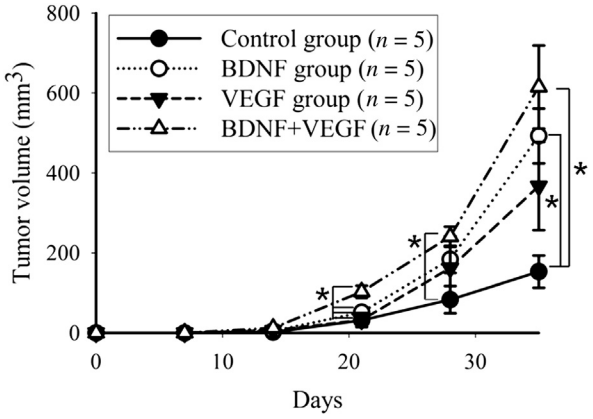

Fig. 3. Exogenous 100-ng brain-derived neurotrophic factor (BDNF) and/or 100-ng vascular endothelial growth factor (VEG) in 50 μL distilled water was injected subcutaneously into the loading site of BFTC905 cells (1×10^6 BFTC905 cells) once a week, starting the day following tumor injection. The xenograft tumor volume in severe combined immunodeficiency mice was measured weekly for 5 weeks. * $p < 0.05$.

growth as shown in Fig. 3. The largest effect was found for BDNF plus VEGF, whereas VEGF exerted the smallest effect. Compared with vehicle injection, BDNF plus VEGF exhibited a significant increase in tumor volume at 21 days after drug treatment, and this drug combination continued to promote the largest tumor growth. Significant growth promotion was observed for BDNF at 35 days after treatment. At the end of the 35-day treatment, mean tumor volumes were 615.2 ± 104.0 mm^3, 492.7 ± 68.6 mm^3, 391.5 ± 127.9 mm^3, and 153.2 ± 40.2 mm^3 in the BDNF + VEGF, BDNF, VEGF, and control groups, respectively.

3.4. No significant difference in tumor volumes or expression of CD34 and Ki-67 in BFTC905 xenografts 6 weeks after BDNF and/or VEGF administration

After the 6-week treatment, the volumes of BFTC905 xenografts reached a mean 739.2 ± 172.0 mm^3, 574.3 ± 89.1 mm^3, 416.2 ± 99.5 mm^3, and 703.0 ± 230.0 mm^3 in the BDNF + VEGF, BDNF, VEGF, and control groups, respectively (Fig. 4A). Following one-way ANOVA and post-tests with Bonferroni multiple comparisons, no significant difference in tumor volumes was observed among the four groups. One small (1 mm in diameter) gross hepatic

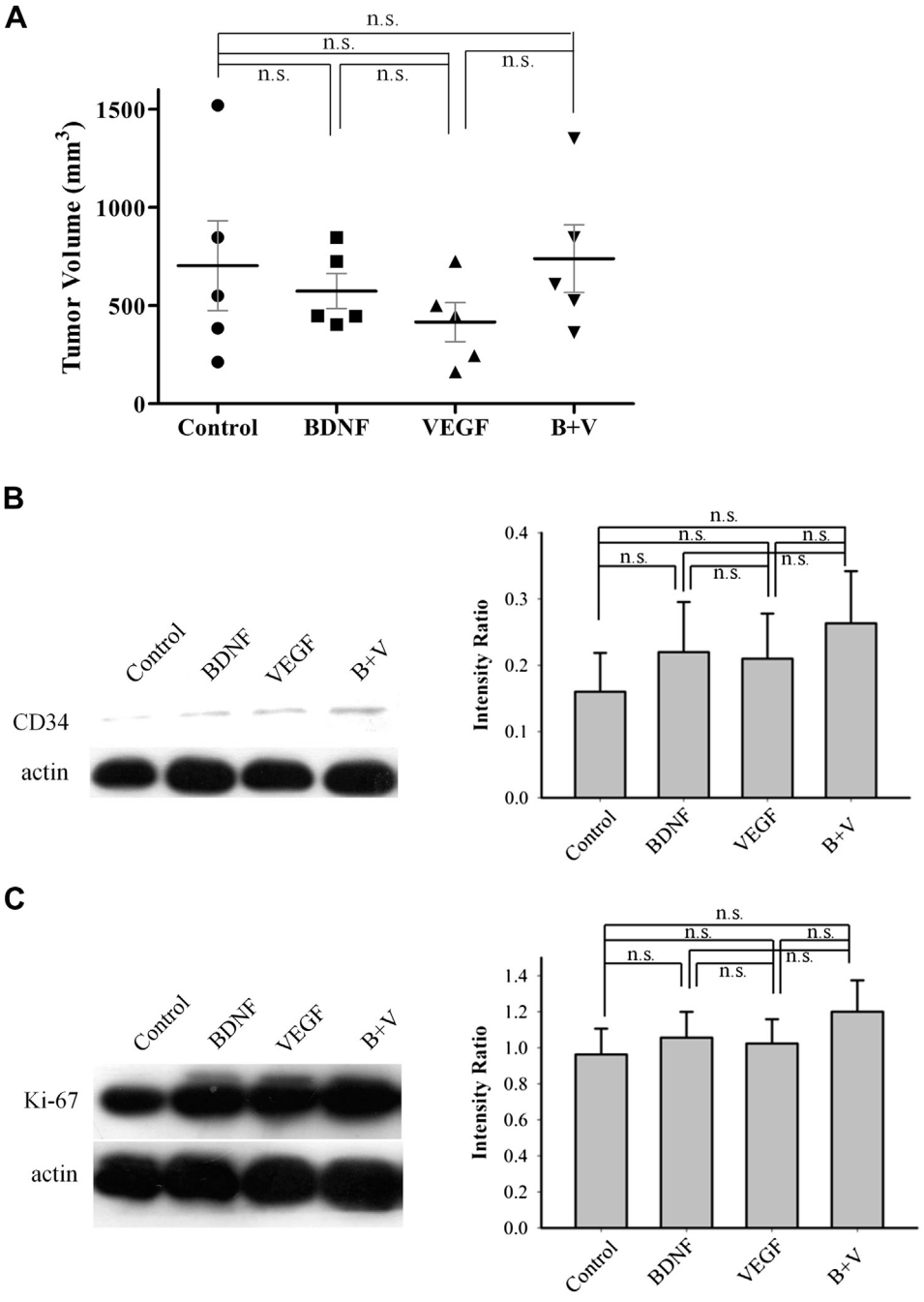

Fig. 4. Volumes and markers for xenograft tumors in severe combined immunodeficiency mice. (A) Xenograft tumor volume measured from mice sacrificed after 6 weeks of treatment. (B) Expression of CD34 after exogenous brain-derived neurotrophic factor (BDNF) and/or vascular endothelial growth factor (VEGF) in BFTC905 xenograft tumors. Values are mean ± standard error of the mean (SEM) from three separate experiments. (C) Expression of Ki-67 after exogenous BDNF and/or VEGF in BFTC905 xenograft tumors. Values are mean ± SEM from three separate experiments. B + V = BDNF + VEGF; n.s. = no significance.

metastasis was found in one mouse in the VEGF group. Another mouse in the VEGF group showed multiple small metastases (1 mm in diameter) over the right lung. Peritoneal seeding was also observed in the two previously mentioned mice in the VEGF group, and one mouse each in the BDNF and VEGF plus BDNF group. No gross metastases were found in the control group.

VEGF has been demonstrated to promote angiogenesis, such as in bladder cancer [18]. In addition, Nakamura et al reported that BDNF induces VEGF expression in neuroblastoma cells [19]. We therefore examined whether the angiogenic effect affected the growth of xenograft tumors after BDNF and/or VEGF administration. CD34 is a marker for angiogenesis in bladder cancer [20] and Ki-67 is a nuclear protein tightly associated with cell proliferation [21]. We found that the expression levels of CD34 and Ki-67 in xenograft tumors were similar among the four experimental groups after the 6-week treatment (Fig. 4B and C).

4. Discussion

In this study, we demonstrated the promoting and additive effect of BDNF plus VEGF on the early growth of the bladder cancer cell line BFTC905 in both a culture system and xenograft tumors. These results suggest that BDNF and VEGF may promote TCC growth through different signaling pathways. Of course, the possibility that both BDNF and VEGF may act on the same pathway cannot be excluded because the dose-response effect of BDNF and VEGF was not established in this study.

BNDF and VEGF did not promote proliferation of primary human urothelial cells as shown by weak immunostaining of their specific receptors TrkB and FLT-1, respectively. This is consistent with our previous report that TrkB is weakly expressed in specimens of normal human urothelium [13]. VEGFR has also been found in specimens of normal human urothelium, and upregulated in inflamed normal rodent urothelial cells [22]. However, no study has investigated the proliferative effects of BDNF and/or VEGF on normal human urothelium. Our preliminary data indicated that BNDF and VEGF did not promote proliferation of primary human urothelial cells.

For the past 20 years, xenograft tumors of human cancer cells implanted subcutaneously into immunosuppressed mice have been widely applied preclinically to evaluate the effect of anticancer drugs. In our previous study, we demonstrated that fewer cells were needed to establish xenograft tumors when the Taiwan TCC cell line BFTC905 was used compared with T24, which is commonly used in bladder cancer research, and another Taiwan TCC cell line, TSGH8301 [12]. After implantation of BFTC905 cells for 6 weeks, some metastases were found. This finding is consistent with a previous report that the limitations of a xenograft model are slow growth and few metastases [23]. Therefore, orthotopic models may be more appropriate for the investigation of tumor progression [24].

After 5 weeks of treatment, the growth of BFTC905 xenograft tumors in severe combined immunodeficient mice was BDNF + VEGF > BDNF > VEGF > controls. The difference was statistically significant. These data are compatible with previous reports that BDNF [12] and VEGF [15] are survival factors for TCCs. However, no statistical difference in tumor sizes was observed 6 weeks after treatment with BDNF and/or VEGF. One possibility is the large variation in tumor volumes found among individual animals. Thus, one limitation of this experiment was the insufficient number of animals used. In addition, metastases were noted after sacrifice, so the original tumor sizes cannot simply reflect the degree of cancer progression. Furthermore, the growth factor is just one of 10 hallmarks of cancer growth [25]. Once neoplastic tumors become uncontrolled growth, as in patients in the terminal stages of cancer, cancer cells can survive and invade via multiple signaling pathways. This may explain why no difference in BFTC905 xenograft tumor sizes was observed after weekly administration of growth factors for 6 weeks in our study.

It is reasonable that the angiogenic marker CD34 in human bladder cancer specimens is considered a prognostic factor for early recurrence [26]. To analyze microvascular density, immunostaining of CD34, a specific marker of endothelial cells, is a common method of evaluation of angiogenesis [27]. We have also examined the localization of CD34 in xenograft tumors by immunofluorescence, but few and scattered distribution of positive CD34 endothelial cells were seen (results not shown). The field we choose in microscopy would influence the results for semiquantification, so Western blotting was used instead to detect the expression of CD34 in xenograft tumors. Low immunostaining of CD34 on Western blotting was correlated with the findings on immunofluorescence, and low CD34 expression may explain the reason for the low number of metastases in this investigation. Thus, exogenous VEGF may act directly on TCC cells to affect the growth of xenograft tumors, and this *in vivo* result coincides with the *in vitro* observations.

Ki-67 protein is present during all active phases of the cell cycle (G_1, S, G_2, and mitotic phase) but absent from resting cells (G_0), so Ki-67 expression in human bladder cancer tissues is significantly greater in high-grade tumors and associated with a significantly worse prognosis [28–30]. Nuclear Ki-67 is usually detected by immunohistochemistry, and additional semiquantification is used to evaluate the proliferative status. We also examined the immunostaining of Ki-67 in xenograft tumors; however, it was difficult to count cells because every field chosen for microscopy among the four experimental groups was almost invariably filled with positive Ki-67 cells (results not shown). Thus, we used Western blotting to detect changes in Ki-67 protein, and no significant difference was observed, as shown by the abundant expression of Ki-67 in the four groups of xenograft tumors. No difference in the expression of Ki-67 is consistent with no difference in the xenograft tumor sizes among the four experimental groups after 6 weeks of treatment.

In conclusion, exogenous BDNF plus VEGF additively promoted the early growth of bladder cancers *in vitro* and *in vivo*, without affecting the normal urothelium. Targeted blockade of both TrkB and VEGF signaling may be an effective treatment for adjuvant postoperative bladder cancer therapy or in the early stage of high-grade TCC when patients refuse surgery.

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