Research Article

Investigation of Antiangiogenic Tumor Therapy Potential of Microencapsulated HEK293 VEGF$_{165}$b Producing Cells

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To investigate the antiangiogenic potential of encapsulated VEGF$_{165}$b producing HEK293 cells, Human Embryonic Kidney 293 (HEK293) cells were stably transfected to produce VEGF$_{165}$b. Then they were encapsulated in alginate - polylysine -alginate (APA) microcapsules. VEGF$_{165}$b productivity and viability of encapsulated cells were analyzed and compared with the non-encapsulated cells. Results showed that encapsulated cells proliferated and remained viable within the microcapsules throughout the 28-day period of the experiment. The quantity of VEGF$_{165}$b increased from $6.5 \pm 1.2\,\mu$g/ml at day 13 to $13 \pm 0.96\,\mu$g/ml at day 16. Then it gradually dropped to $5 \pm 1.2\,\mu$g/ml for the last 3 days period as measured at day 28. Production of VEGF$_{165}$b from encapsulated and non-encapsulated cells was similar. The effect of VEGF$_{165}$b harvested from encapsulated cells on Human Umbilical Vein Endothelial cells (HUVECs) proliferation were also examined. The same inhibitory effects on HUVECs proliferation was seen when the cells were incubated with a mixture of VEGF$_{165}$b and a 2-fold VEGF$_{165}$b or with VEGF$_{165}$b and 2-fold excess VEGF$_{165}$b released from encapsulated cells. Subcutaneous injection of microencapsulated VEGF$_{165}$b producing cells in tumor site of nude mice resulted in the reduction of the number of vessels around the tumors.

1. Introduction

Vascular endothelial growth factor (VEGF) is a prominent growth factor and a key regulator of normal and patho-physiological angiogenesis [1, 2]. VEGF is a multifunctional glycoprotein that stimulates endothelial cell migration and proliferation, increases vascular permeability, and protects endothelial cells from apoptosis [3]. VEGF expression is upregulated in the majority of human cancers and it is considered as an important target for anticancer therapy [4, 5]. Overexpression of angiogenic factors generates the imbalance between angiogenesis regulators and inhibitors and stimulates endothelial cell activation which leads to angiogenic switch and consequently allows the tumor to access a nutrition source essential for its growth and metastasis [6, 7].

Among VEGF isoforms, VEGF$_{165}$ is the most commonly detected isoform and the predominant growth factor and plays a fundamental role in tumor angiogenesis [8]. The angiogenesis effects of VEGF$_{165}$ appear to be mediated mainly by activation of VEGF receptor 2 (VEGFR-2). VEGF$_{165}$ can be inhibited by its own counterpart VEGF$_{165}$b. VEGF$_{165}$b is one of VEGF isoforms that inhibits VEGF$_{165}$-mediated signaling by acting as a competitive antagonist of VEGFR2. VEGF$_{165}$b does not stimulate VEGFR-2 phosphorylation in the same manner as VEGF$_{165}$ and inhibits VEGF-dependent angiogenesis. Hence, this VEGF isoform can be a potential therapeutic protein for antiangiogenic therapy in tumor suppression [9–12]. To develop a system, continuously producing this protein, we considered using engineered cells to supply a stable source of protein, encapsulate the engineered cells to protect them from host immune system and implant the encapsulated producing cells *in vivo*. Microencapsulation of cells provides a safe environment for the recombinant cells to be able to survive, proliferate, and produce the desired protein in spite of the

presence of host immune mediators, which are responsible for graft rejection [13, 14]. This protection is achievable via a permselective membrane of the microcapsule that eliminates effects of host immune system and provides free access of nutrients and oxygen for implanted encapsulated cells [15, 16]. We hypothesized that microencapsulation of engineered cells stably producing VEGF$_{165}$b and implanting the encapsulated cells in tumor-site would provide clinical benefits by reducing tumor mediated angiogenesis and preventing the tumor converting from a dormant lesion to a rapidly enlarging metastatic mass [17].

In this study, for the first time, APA microencapsulated VEGF$_{165}$b producing HEK293 cells have been used to provide continuous source of VEGF$_{165}$b for antiangiogenic therapy using various *in vitro* procedures. The antiangiogenesis preclinical efficacy of VEGF$_{165}$b released from microcapsules containing HEK293 VEGF$_{165}$b producing cells was investigated in nude mice.

2. Materials and Methods

2.1. Cell Line. The human embryonic kidney 293 cell line, stably expressing Epstein-Barr virus Nuclear Antigen-1 (clone 6E), was grown as a suspension culture in FreeStyle or Freestyle-F17 (F17; Invitrogen) medium, a serum- and protein-free medium, supplemented with 0.1% pluronic F-68 and 50 μg/ml Geneticin. Cells were grown agitated at 110 rpm at 37°C in a humidified 5% CO$_2$ chamber and routinely passed in 125-ml Erlenmeyer flasks containing 20 ml of culture medium.

An ELISA was carried out to measure the quantity of VEGF$_{165}$b harvested from encapsulated VEGF$_{165}$b producing cells and nonencapsulated VEGF$_{165}$b producing cells. The quantity of VEGF$_{165}$b increased from 6.5 ± 1.2 μg/ml at day 13 to 13 ± 0.96 μg/ml at day 16. The level of VEGF$_{165}$b then gradually dropped to 5 ± 1.2 μg/ml for the last 3 days period. The highest quantity of VEGF$_{165}$b secreted from nonencapsulated cells was 14.2 ± 0.84 and 15 ± 1.8 at day 16 and 19, respectively. The values then dropped to 7 ± 0.8 at day 28 (Figure 3).

2.2. Plasmid Construction and Purification. The human codon-optimized VEGF$_{165}$b cDNA (Geneart) was inserted between the EcoRI and BamHI sites of the pYD7 vector (pTT vector bearing a blasticidin resistance expression cassette) [18–20]. E. coli DH5α grown in Circle Growth broth supplemented with ampicillin (100 μg/ml) was used to amplify the plasmid, which was purified using a Maxiprep plasmid purification kit (Qiagen, Valencia, CA). Plasmid DNA concentration was measured at 260 nm following its dilution in 50 mM Tris-HCl, pH 8.0. Plasmid DNA was then linearized by PvuI digestion for stable integration into chromosomal DNA.

2.3. Transfection, Selection, and Clone Isolation. The transfection method was performed using previously established procedure [18, 21]. Briefly, the cells were seeded at 1×10^6 cells/ml before transfection. DNA was diluted in fresh serum-free medium in a volume of one-tenth of the culture to be transfected at a concentration of 1.0 μg/ml. PEI [22] (branched 25 kDa, Aldrich) was added at a ratio of 1 : 1.5 (DNA : PEI). The mixture was vortexed and incubated at room temperature for 15 minutes. It was then added to the cells in a Nunclon Surface plate and incubated at 37°C and agitated at 110 rpm for one hour. The cells were then plated as 0.3×10^6 cells/well in a 6-well CellBind plate and incubated at 37°C. Blasticidin was added at 24 hours post transfection at a concentration of 1 μg/ml. After 2 weeks of selection with regular medium changes, blasticidin-resistant cells were plated in CellBind 96-well plates at a density of 1 cell per well in 200 μl FreeStyle media supplemented with 50% conditioned media, 0.05% TN1, and phenol red. Each well was visually examined the day after plating and those containing a single cell were identified. Half of the medium was replaced by fresh medium every 10 days. After 20–30 days, the presence of VEGF$_{165}$b in the culture medium was analyzed by western blot. The wells containing the cells producing high amount of VEGF$_{165}$b were identified and the cells were amplified. In order to select the best clone, cells were seeded in a 6-well plate at the concentration of 0.5×10^6 cells/well and incubated at 37°C in a humidified 5% CO$_2$ chamber. Five days later, the cells were counted and the supernatant was analyzed by western blot for the presence of VEGF$_{165}$b. The clone with best growth rate and highest amount of protein production was selected (clone F10).

2.4. Microencapsulation of Transfected Cells. Microencapsulation of HEK293 VEGF$_{165}$b producing cells (clone F10) was performed as described previously with slight modification [23–27]. Cells were centrifuged at 1000 g for 10 min. The supernatant was decanted and the cells were resuspended in 25 ± 5 ml sterile-filtered 1.65% (w/v) alginate (obtained from Sigma-Aldrich, low viscosity) solution in 0.85% (w/v) saline to give 1×10^6 cells/ml. The mixture of cells and alginate solution was loaded in a 60 ml syringe and extruded through an Inotech Encapsulator IER-20 (Inotech Biosystems International, Rockville, MD) with a 300 μm nozzle at a frequency of 1052 Hz and a voltage of 1.0 kV. Alginate droplets were collected in 1.5% CaCl$_2$ solution and the gelation process took place for 30 minutes. To prepare APA microcapsules, Ca-alginate beads were immersed in a 0.1% (w/v) poly-l-lysine (Sigma, MW 27400) solution in 0.85% (w/v) saline for 15 minutes followed by a 10-minute incubation in a 0.1% (w/v) alginate solution in 0.85% (w/v) saline. The final microcapsules were transferred to the same media used for nonencapsulated cells and stored at 37°C for further analysis.

2.5. Monitoring Metabolic Activity of Encapsulated Cells. The metabolic activity and cell proliferation of encapsulated cells were determined using an MTS colorimetric assay (Promega). The assay is based on the ability of dehydrogenase enzymes found in metabolically active cells to convert MTS into a formazan product that is soluble in culture medium. The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture.

The assay was performed according to previously described procedures with slight modifications [28–30]. Every three days, approximately 50 ± 5 microcapsules were transferred to a 96-well plate and incubated with $100\,\mu l$ media and $20\,\mu l$ MTS/PMS solution at $37°C$ for 4 hours. The optical density was read and recorded at 490 nm using a plate reader. The cells were quantified using a calibration curve correlating cell number with absorbance. The metabolic activity of nonencapsulated cells was also determined using the same protocol. An equivalent number of nonencapsulated cells were plated in a 96-well plate and every three days their metabolic activity was determined. The cell number was obtained using a calibration curve correlating cell quantity with absorbance.

2.6. Western Blot Analysis of Protein Productivity of Encapsulated Cells. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot were performed according to standard procedure to verify the protein production of microencapsulated 293/IFNα cells. Encapsulated cells were suspended in F17 medium and incubated at $37°C$ in a humidified 5% CO_2 chamber. The medium was replaced every three days and analyzed by western blot for the presence of VEGF$_{165}$b. The harvested medium from encapsulated cells was diluted in NuPAGE 4X sample buffer (Invitrogen, Carlsbad, CA) containing 50 mM DTT and then heated at $70°C$ for 10 min. Separation was performed on NuPAGE 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA) using MES running buffer for 40 min at 200 V. Western blots were performed by transferring proteins to nitrocellulose membrane using Tris-glycine buffer for 1 hour at 300 mA. The membrane was then incubated with a rabbit antihuman VEGF (R&D) diluted 1 : 500 for 1 hour followed by incubation with an antirabbit horseradish peroxidase (1 : 5000) for 1 hour. The blots were revealed using a BM Chemiluminescent Blotting kit (Roche). The same procedure was performed for free nonencapsulated cells to compare VEGF$_{165}$b productivity to encapsulated cells by plating an equivalent number of cells in a 96-well plate. The medium was replaced every three days and analyzed by western blot for the presence of VEGF$_{165}$b.

2.7. VEGF$_{165}$b Quantification. The VEGF$_{165}$b concentration in conditioned media of encapsulated cells was determined with an enzyme-linked immunosorbent assay (ELISA) following the protocol supplied by the Human VEGF ELISA kit (DVE00, R&D).

2.8. In Vitro Bioactivity Assay of VEGF$_{165}$b, HUVECs Proliferation. The effects of VEGF and VEGF$_{165}$b on HUVECs proliferation were evaluated as described previously [31–33]. HUVECs were seeded as 5000 cells/well in a 96-well plate. The cells were serum- and growth factors-starved overnight. The cells were then divided into 3 groups, one group received different concentration of VEGF, and the other two groups received VEGF with two-fold dilution series of either purified VEGF$_{165}$b or VEGF$_{165}$b collected from supernatant of the encapsulated cells. HUVEC proliferation was determined after 72 hours by MTS-based assay.

2.9. In Vivo Study of the Antiangiogenesis Effects of VEGF$_{165}$b. To verify the effects of VEGF$_{165}$b on angiogenesis, 10^5 Tpr-Met Fr3T3 fibroblast cells mixed with $250\,\mu l$ of serum-depleted Matrigel (Becton Dickinson Labware, Bedford, MA) were injected subcutaneously on the flanks of 5-week-old female nude mice (Charles River Breeding Laboratories). The mice were divided into three groups. One group received 70 ± 10 VEGF$_{165}$b/293 microcapsules per $100\,\mu l$ of PBS. The second group received 70 ± 10 parental/293 microcapsules per $100\,\mu l$ of PBS and the last group were injected $100\,\mu l$ of PBS. The microcapsules were injected at the same side as matrigel plug. After 9 days, the mice were sacrificed and Matrigel plugs were retrieved from the animals to visualize the presence of microvessels.

3. Results

3.1. Expression of VEGF$_{165}$b from Transfected Cells. Transfected cells were clone isolated as described above. The clones were analyzed for VEGF$_{165}$b production by western blot and SDS-PAGE electrophoresis. Five positive clones were selected from screening 35 clones. They all had positive bands with various intensity running at approximately around 30 kDa as expected for VEGF$_{165}$b. The final verification of protein expression of isolated clones was performed using ELISA kit and the clone with highest protein production was selected (clone F10).

3.2. Morphological Studies and Viability of Encapsulated Cells. Results showed that microcapsules with a diameter of $500 \pm 50\,\mu m$ could be prepared. The cells were evenly distributed within the microcapsules. The cells created colonies within the microcapsules and then the colonies enlarged and filled up most of the interior space of the microcapsules. Growth and viability of encapsulated cells were analyzed using MTS assay. Results showed that the encapsulated cells grew over a 13–16-day period after microencapsulation, at this time the viable cell density reached approximately $1 \times 10^4 \pm 1000$/microcapsule. Then, the viability of encapsulated cells decreased slowly until day 28 of the experiment. The viability of encapsulated cells was compared with nonencapsulated cells. Nonencapsulated cells showed similar growth pattern to encapsulated cells. Nonencapsulated cells proliferated from day first to day 16. The viability of nonencapsulated cells decreased slowly thereafter (Figure 1).

3.3. In Vitro Expression and Release of VEGF$_{165}$b from Encapsulated Cells. Experiments were designed to investigate production of VEGF$_{165}$b by encapsulated HEK293 VEGF$_{165}$b producing cells.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot were performed to analyze the production of VEGF$_{165}$b by encapsulated cells. The medium was harvested and fresh medium was added every three days. The results showed that the production of VEGF$_{165}$b was detected 4 days after encapsulation and had the highest production at day 16. The pick of VEGF$_{165}$b production at day 16 was compatible with the cell growth curve.
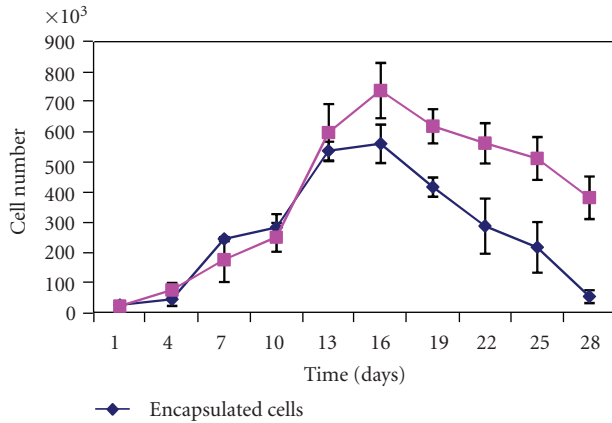
FIGURE 1: The number of viable encapsulated and nonencapsulated HEK293 VEGF$_{165}$b producing cells determined by MTS assay.
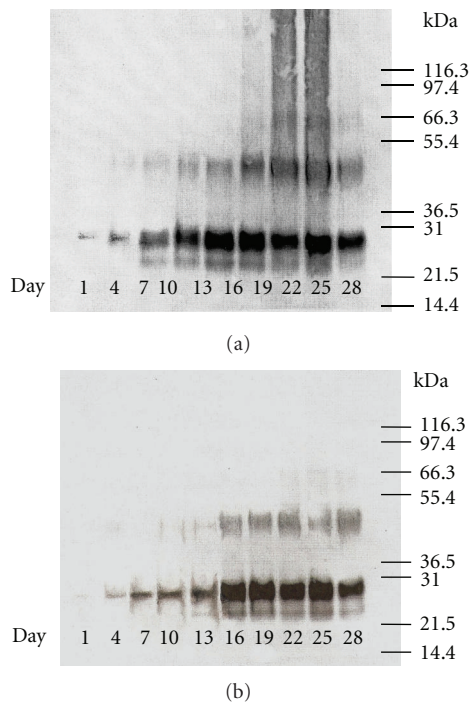

(a)

(b)

FIGURE 2: Western blot analysis of human VEGF$_{165}$b produced by (a) encapsulated HEK293 VEGF$_{165}$b producing cells and (b) nonencapsulated HEK293 VEGF$_{165}$b producing cells.

FIGURE 3: The quantity of secreted VEGF$_{165}$b from encapsulated and nonencapsulated HEK293 VEGF$_{165}$b producing cells determined by ELISA.
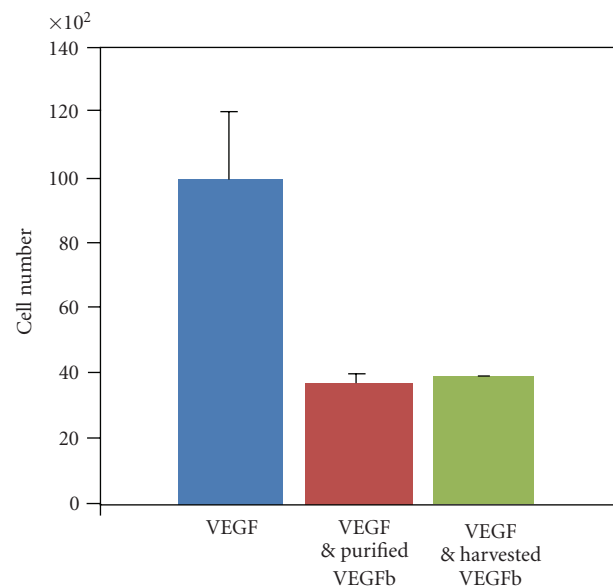
FIGURE 4: The effects of VEGF and VEGF$_{165}$b on HUVECs proliferation. The cells received 25 ng/ml VEGF, 25 ng/ml VEGF and two-fold purified VEGF$_{165}$b or 25 ng/ml VEGF and two-fold harvested VEGF$_{165}$b from microencapsulated HEK293 VEGF$_{165}$b producing cells after overnight starving from serum and growth factors. HUVEC proliferation was determined after 72 hours by MTS-based assay. Purified VEGF$_{165}$b and harvested VEGF$_{165}$b from encapsulated HEK293 VEGF$_{165}$b producing cells offered similar results in inhibition of cell proliferation, $n = 3$, $P = .06$.

Production of VEGF$_{165}$b then decreased between days 19 and 28. Production of VEGF$_{165}$b from nonencapsulated cells followed similar pattern to that of encapsulated cells (Figure 2).

An ELISA was carried out to measure the quantity of VEGF$_{165}$b harvested from encapsulated VEGF$_{165}$b producing cells and nonencapsulated VEGF$_{165}$b producing cells. The quantity of VEGF$_{165}$b increased from $6.5 \pm 1.2 \, \mu g/ml$ at day 13 to $13 \pm 0.96 \, \mu g/ml$ at day 16. The level of VEGF$_{165}$b then gradually dropped to $5 \pm 1.2 \, \mu g/ml$ for the last 3 days period. The highest quantity of VEGF$_{165}$b secreted from nonencapsulated cells was 14.2 ± 0.84 and 15 ± 1.8 at day 16 and 19 respectively. The values then dropped to 7 ± 0.8 at day 28 (Figure 3).
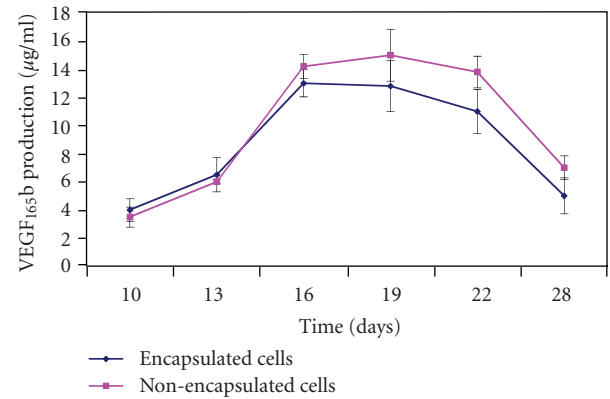
3.4. In Vitro Bioactivity Assay of VEGF$_{165}$b Expressed from Encapsulated Cells.
The effects of human VEGF$_{165}$b produced by encapsulated cells on endothelial cells were evaluated using HUVECs proliferation assay. Results showed that addition of VEGF to the cells enhanced HUVEC proliferation and viability in a dose-dependent manner. VEGF$_{165}$b produced by encapsulated and nonencapsulated cells prevented cell proliferation with the presence of VEGF. In the control group of cells in which no VEGF was added, no viability was observed. The highest endothelial cell proliferation was
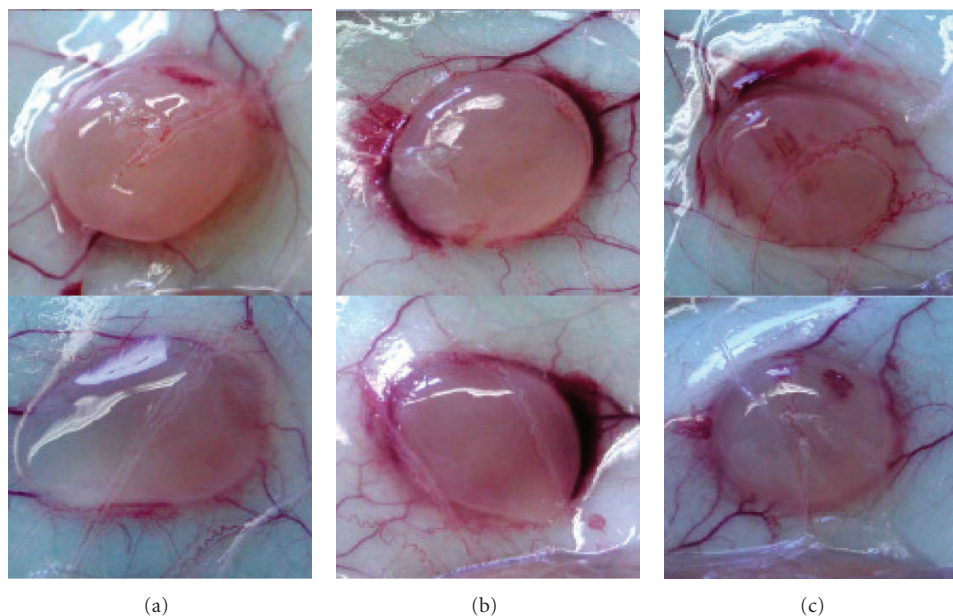
FIGURE 5: Tumor angiogenesis effects microencapsulated HEK293 VEGF$_{165}$b producing cells in experimental nude mice. Top and bottom reprentative sample of (a) Matrigel plugs with microcapsules containing HEK293 VEGF$_{165}$b producing cells, (b) Matrigel plugs with microcapsules containing parental HEK293 cells, and (c) matrigel plugs with vehicle (PBS).

observed by adding 25 ng/ml VEGF. At this point, purified VEGF$_{165}$b, and harvested VEGF$_{165}$b presented the highest antagonistic effects. The number of cells lessened from $1 \times 10^4 \pm 2000$ cells with VEGF to 3700 ± 140 cells with VEGF and purified VEGF$_{165}$b and to 3900 ± 50 cells with VEGF and harvested VEGF$_{165}$b from encapsulated cells. Higher concentration of VEGF from 50 to 200 ng/ml had less effect on increasing HUVECs proliferation. Similarly HUVECs proliferation was less affected with higher concentration of VEGF$_{165}$b, 400 ng/ml. Purified VEGF$_{165}$b and harvested VEGF$_{165}$b from encapsulated cells offered similar results in inhibition of cell proliferation (Figure 4).

3.5. Inhibition of Angiogenesis by VEGF$_{165}$b. The in vivo experiment was designed to observe the effects of VEGF$_{165}$b produced by encapsulated cells on angiogenesis in tumors. Tumor cells mixed with matrigel were s.c. injected to nude mice as described above. Photographs of retrieved matrigel plugs from animals showed tumor angiogenesis (Figure 5). Use of encapsulated VEGF$_{165}$b producing cells in tumor site significantly decreased total vascular density. The number of vessels around the tumor with microcapsules containing VEGF$_{165}$b producing cells reduced compared to the ones with microcapsules containing parental HEK293 cells and matrigel control vehicle, which indicated the release of VEGF$_{165}$b from encapsulated cells and effects of VEGF$_{165}$b on prevention of angiogenesis.

4. Discussion

Inhibition of angiogenesis has been broadly documented as a promising approach for cancer treatment [34]. This therapy offers several advantages over the conventional cancer therapy. For instance, one approved angiogenesis inhibitor can be used in different types of tumors, as solid tumors are angiogenesis dependent. Antiangiogenesis targets endothelial cells, which are genetically stable compared to tumor cells, therefore, drug resistant occurs rarely. Furthermore, it has fewer systemic side effects since angiogenesis has limited actions in adults. To establish an efficient angiogenesis therapy, recently different strategies have been studied to block VEGF pathway. VEGF is upregulated in the majority of human cancers, so it is known as a valid target for antiangiogenic therapy [6]. This certainty has led the cancer research to focus on the development of the drugs inhibiting VEGF activity [3, 35]. In this study, we investigated the efficacy of the encapsulated-producing cells for providing constant release of VEGF$_{165}$b. VEGF$_{165}$b binds to VEGFR2, the main VEGF receptor in angiogenesis; therefore it can narrowly target angiogenesis activation in tumor.

VEGF$_{165}$b is considered as an endogenous angiogenesis inhibitor as it can be expressed in normal human tissues. Increasing endogenous inhibitors has been appeared to be a safe and reliable approach in long-term cancer therapy [36]. For example, overexpression of endostatin, an endogenous angiogenesis inhibitor with broad spectrum has appeared to slow down tumor growth in mice [34, 37]. Overexpression of VEGF$_{165}$b has been also shown to inhibit growth of sarcoma, renal, prostate [38], and colorectal cancer in mice [39].

In the current study, the efficacy of encapsulated VEGF$_{165}$b producing cells for antiangiogenesis therapy was evaluated. Encapsulation of producing cells promises providing the sustained release of the protein required for long-term treatments in tumor suppression [40]. Slow release of protein from engineered cells via encapsulation has

been applied in various diseases including antiangiogenic cancer therapy [41–43]. Microencapsulation of $VEGF_{165}b$ producing cells showed that the cells were able to survive and release protein throughout the period of the experiment providing the slow release of $VEGF_{165}b$. The bioactivity of the released protein on HUVECs proliferation was also studied. HUVECs proliferation was clearly promoted in the presence of VEGF. The highest effect of VEGF was observed at the concentration of 25 ng/ml. At this concentration of VEGF, $VEGF_{165}b$ harvested from encapsulated cells offered approximately highest proliferation inhibition. Being a competitive antagonist of VEGF, the concentration of $VEGF_{165}b$ at each point was two-fold more than VEGF to induce the antagonistic effects. Furthermore, reducing microvessels in tumor site after injection of microencapsulated $VEGF_{165}b$ producing cells in animals verified the efficacy of this system to maintain the cells alive and able to produce the desired protein *in vivo*. Although these finding are encouraging in cancer treatment, there are still numerous unanswered questions that have to be revealed in order to obtain a practical approach to suppress tumors including the accurate dosage of an angiogenic inhibitor, the length of treatment, and the efficacy of combination therapy that need further analysis.

Delivery of $VEGF_{165}b$ by encapsulated HEK293 $VEGF_{165}b$ producing cells reveals the potential of cell microencapsulation for sustained release of $VEGF_{165}b$. Cell microencapsulation can be applied for continuous release of bioactive therapeutic proteins for medical purposes.

Acknowledgments

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