

## A novel gene delivery system targeting cells expressing VEGF receptors\*

LI Jun Min<sup>1\*\*</sup>, Jun Song HAN<sup>1</sup>, Yi HUANG<sup>1</sup>, Pei Kun TIAN<sup>1</sup> Shu Min QU<sup>1</sup>, Min YAO<sup>1</sup>, Hui Qiu JIANG<sup>1</sup>, Da Fang WAN<sup>1</sup>, Jing Chu LUO<sup>2</sup>, Cheng Xiao GU<sup>2</sup>, Jian Ren GU<sup>1\*\*\*</sup>

<sup>1</sup> National Laboratory for Oncogenes and Related Genes, Shanghai Cancer Institute, Shanghai 200032, China

<sup>2</sup> National Laboratory of Protein Engineering and Plant Genetic Engineering, Peking University, Beijing 100871, China

### ABSTRACT

Two ligand oligopeptides GV1 and GV2 were designed according to the putative binding region of VEGF to its receptors. GV1, GV2 and endosome releasing oligopeptide HA20 were conjugated with poly-L-lysine or protamine and the resulting conjugates could interact with DNA in a noncovalent bond to form a complex. Using pSV2- $\beta$ -galactosidase as a reporter gene, it has been demonstrated that exogenous gene was transferred into bovine aortic arch-derived endothelial cells (ABAE) and human malignant melanoma cell lines (A375) *in vitro*. *In vivo* experiments, exogenous gene was transferred into tumor vascular endothelial cells and tumor cells of subcutaneously transplanted human colon cancer LOVO, human malignant melanoma A375 and human hepatoma graft in nude mice. This system could also target gene to intrahepatically transplanted human hepatoma injected via portal vein in nude mice. These results are correlated with the

---

\* The present receptor-mediated gene delivery system has been patented by Gu and Tian (Patent Registration number in China 96116557 X; PCT/CN 97/00106)

\*\* The present address of the author is Zhejiang Taizhou Normal College, Zhejiang 317000, China.

\*\*\* Corresponding author, Fax: 021-64177401, E-mail: nlorg@public.sta.net.cn

Gene delivery system targeting VEGF receptors

relevant receptors (flt-1, flk-1/KDR) expression on the targeted cells and tissues.

**Key words:** *VEGF receptors, gene delivery system, tumor, vascular endothelial cells, targeting.*

## INTRODUCTION

Receptor-mediated gene transfer system utilized complexes of DNA with ligands such as transferrin or asialoglycoprotein to deliver exogenous genes into cells with relevant receptor expression[1-2]. In our laboratory, IE5 and GE7 ligand oligopeptide (LOP) were successfully developed to deliver exogenous genes into tumor cells with IGF II or IGF I receptor and EGF receptor expression[3]. The DNA-polycation-ligand complex utilized receptor mediated endocytosis to deliver the gene into the targeted cells[4].

Angiogenesis is one of the most important features in the progression of cancer. The growth of solid tumors and the formation of metastases are dependent on the formation of new vasculatures[5]. The mechanism supporting the angiogenesis in tumor remains unknown. Among the various angiogenic factors, vascular endothelial growth factor (VEGF) plays a pivotal role in tumor angiogenesis[6]. Biological actions of VEGF are mediated through specific tyrosine kinase receptors with high affinity. VEGF is expressed mainly in tumor cells, associated with angiogenesis and metastasis[7]. In most solid tumors, VEGF receptors expression were up-regulated in tumor vascular endothelial cells accompanying the up-regulation of VEGF expression in tumor cells[8]. VEGF receptors also exist in some invasive tumor cells[9, 10]. In normal tissue, vascular growth is ceased and VEGF receptor are not expressed or only expressed at low level[11, 12]. These properties imply that targetable gene delivery system mediated by VEGF receptors could transfer genes to tumor vascular endothelial cells or some tumor cells with VEGF receptor expression, and would not influence the growth of normal cells.

Two ligand oligopeptides (LOP) GV1 and GV2 were designed and synthesized according to the putative binding region of VEGF to its receptors. HA20, a homologue of N-terminus domain of haemagglutinin of Influenza viral envelope protein was synthesized as an endosome-releasing oligopeptide (EROP). GV1, GV2 and HA20 were conjugated with poly-L-lysine or protamine by using a heterobifunctional cross-linking agent N-succinimidyl-3-(2-Pyridyldithio) propionate (SPDP). Poly-L-lysine and protamine were polycation peptides (PCP), so the resulting conjugates could interact with DNA in a noncovalent bond to form a soluble complex designated as GV1 or GV2 gene delivery system. In this paper, we used  $\beta$ -galactosidase gene as a reporter gene and demonstrated that GV1 and GV2 gene delivery system could targetably deliver exogenous gene to the tumor vascular endothelial cells or some

tumor cells with VEGF receptor expression in vitro and in vivo.

## MATERIALS AND METHODS

### *Chemicals*

GV1 (CHPIETLVDIFQEYPDEIEYIFKPSVPLMRP), GV2 (PVPTEESNITMQIMRIKPHQ GQHIGEMSFLQHNKCE) and HA20 (GLFEAIAEFIEGG WEGLEG) were synthesized in Applied Biosystem A430 Peptide synthesizer. All the oligopeptides were purified by High Performance Liquid Chromatography (HPLC) by using C8 reverse phase column chromatography, 10 mm×250 mm (ABI). Poly-L-Lysine with molecular weight 26KD was purchased from Sigma. Protamine Sulfate with molecular weight 7KD was purchased from Shanghai First Biochemistry Medicine Company. SPDP, Dithiothreitol (DTT), 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactosidase (X-gal) were purchased from Sigma. Sephadex G-25 and Sephadex G-50 were purchased from Pharmacia.

### *Cells and cultures*

Human malignant melanoma cell line A375 was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences and maintained in Dulbecco's modified Eagles medium (GIBCO-BRL) containing 10% fetal calf serum (FCS). Bovine aortic arch-derived endothelial cell (ABAE) was obtained from Institute of Medical Radiology, Shanghai Medical University. Primary cultured ABAE cells were obtained from normal bovine heart aortic arch and maintained in M199 containing 10% fetal calf serum as previously described[13].

### *Animals*

Nude mice with subcutaneously (s.c.) transplants derived from different human cancers such as colon cancer LOVO, malignant melanoma A375 and hepatoma graft were provided by Shanghai Cancer Institute. Nude mice were injected s.c. with  $2 \times 10^6$  cells and ready for use when tumor size reached 0.5 cm in diameter. Highly metastatic transplantable tumor model in nude mice was provided by Dr. Z.Y. Tang's laboratory, Institute of Hepatic Cancer, Zhong Shan Hospital, Shanghai Medical University.

### *Preparation of LOP-PCP and EROP-PCP conjugates*

PCP and SPDP were mixed according to a molar ratio of 1:5, diluted with 0.1 M NaCl-0.1 M phosphate sodium buffer at pH 7.4 to a concentration of PCP at 1 mg/ml. Reaction was performed at 25°C for 2 h. The product PCP-PDP was purified with Sephadex G-25 column (1.6cm × 0cm) chromatography by elution with above buffer so as to remove the residual SPDP. LOP was mixed with PCP-PDP at a molar ratio of 1:1. The reaction was carried out at 25°C for 24 h. LOP-PCP was purified with Sephadex G-50 column chromatography (1 cm × 100 cm) by elution with deionized H<sub>2</sub>O. The product LOP-PCP was concentrated and quantitated by UV spectrophotometry. PCP-PDP was reacted with excess amount of DTT (20 mM) at 25°C for 40 min to produce PCPSH. The product was purified with Sephadex G-25 column chromatography (1.6cm × 50cm) to remove residual DTT by elution with above buffer. EROP-PDP was prepared as described above. EROP-PDP was mixed with PCP-SH at a molar ratio of 1:1. Reaction was performed at 25°C for 72 h. The product EROP-PCP was purified with methods as mentioned above.

### *Determination of the optimal ratio to prepare polypeptide/DNA complex*

Different amount of LOP-PCP and EROP-PCP conjugate were independently mixed dropwise with a given quantity of pSV2- $\beta$ -galactosidase plasmid DNA. The polypeptide/DNA mixture was reacted at 25 °C for 30 min. Mixtures containing 0.2  $\mu$ g plasmid DNA were analyzed with 1% agarose gel electrophoresis to examine the retardation of DNA migration.

## Gene delivery system targeting VEGF receptors

### Preparation of 4-element complex of GV1/PCP/HA20/DNA or GV2/PCP/HA20/DNA gene delivery system and 5-element complex of GV1/GV2/PCP/HA20/DNA gene delivery system

The plasmid pSV2- $\beta$ -gal was purchased from Promega. DNA was prepared by QIAGEN Kit according to the manufacturer's protocol. GV1-PCP, GV2-PCP, HA20-PCP and plasmid DNA were sterilized by filtration through 0.22  $\mu$ m filters (Millipore). Plasmid DNA was dissolved in small aliquot of distilled water. According to the optimal ratio of DNA to conjugates based on the previous experiments, EROP-PCP conjugate was added dropwise to the DNA solution and reacted at 25 °C for 5 min. GV1-PCP or/and GV2-PCP conjugate was then added dropwise with constant stirring. Reaction was carried out at 25 °C for 30 min and then diluted with normal saline. Examine the retardation of DNA by 1% agarose gel electrophoresis.

### *Gene transfer and expression observed in cultured cells*

$2 \times 10^4$  cells were seeded into 1 ml medium each well of 6 well plate. After cell density reached a confluence of about 50%, the medium was removed and replaced with 1 ml of medium containing 4-element complex or 5-element complex with the quantity equivalent to 0.2  $\mu$ g DNA. Cells were cultured at 37 °C and incubated with 5% CO<sub>2</sub> for 24 h, then replaced with fresh medium without polypeptide/DNA complex and cultured at 37 °C with 5% CO<sub>2</sub>. Assay for  $\beta$ -galactosidase expression of cells was carried out at the sixth day.

### *Cytochemical staining of $\beta$ -galactosidase*

Cells were washed twice with 0.1 mol/L PBS (pH 7.4), fixed in a solution of 2% formaldehyde-0.2% glutaraldehyde at room temperature for 10 min, and washed twice with 0.1 M PBS (pH 7.4). Add 1 ml X-gal solution [1 mg/ml X-gal, 5 mM potassium ferrocyanate K<sub>3</sub>(Fe(CN)<sub>6</sub>), 5 mM potassium ferrocyanate K<sub>4</sub>(Fe(CN)<sub>6</sub>), 2 mM MgCl<sub>2</sub>], and react overnight at 37 °C. Calculate the percentage of positive X-gal stained cells under microscope as the efficiency of gene delivery.

### *In vivo gene transduction to nude mice transplanted subcutaneously (s.c.) with cancer cell lines*

Human melanoma cell A375 and other cancer cells were transplanted subcutaneously into nude mice. After tumor size reached about 0.5 cm in diameter, GV1 or GV2 4-element complex was injected s.c. at a dose equivalent to 0.2  $\mu$ g DNA per mouse. Animals were sacrificed 48 h after injection and tumor, heart, liver, spleen, lung, kidney, brain, stomach, small intestine, colon, testis were dissected and washed twice with 0.1 M PBS (pH 7.4). Tissues were fixed in 2% formaldehyde-0.2% glutaraldehyde for 30 min, washed three times with 0.1M PBS (pH 7.4) and stained with X-gal reaction solution at 37 °C for 24 h. Frozen sections were counterstained with Nuclear Fast Red and examined under microscope.

### *In vivo gene transduction mediated by GV2/protamine /HA20/ $\beta$ -gal 4-element complex to human hepatoma intrahepatically transplanted in nude mice*

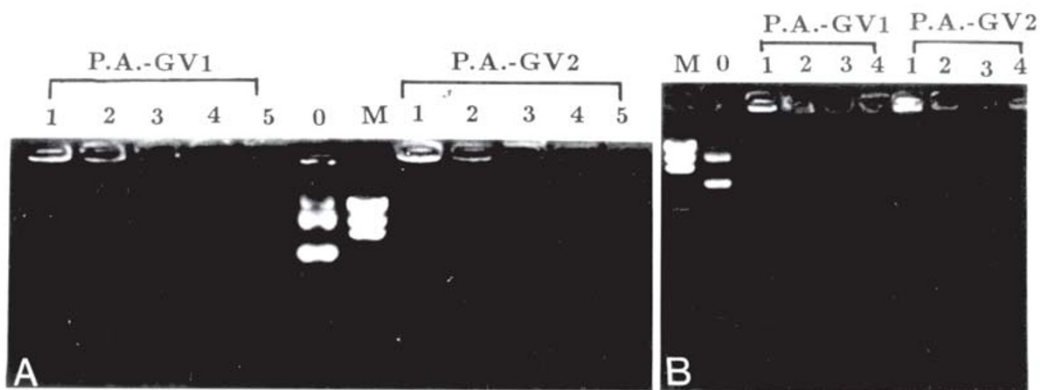
50  $\mu$ l GV2 4-element complex containing 0.5 $\mu$ g pSV2  $\beta$ -gal DNA were injected into portal vein of mice in which human hepatic cancer was transplanted in liver. Animals were sacrificed 14 d after treatment. Tissues were dissected and stained with X-gal as above. Frozen sections were counterstained with Hematoxyln or Fast Nuclear Red and examined under microscope.

## RESULTS

### *Construction of gene delivery system*

GV1, GV2 and HA20 were ligated to protamine or poly-L-lysine by disulfide

bonds. The resulting conjugates could bind DNA by a strong, electrostatic interaction to form a soluble complex. The optimal ratio of  $\beta$ -gal plasmid DNA to protamine and poly-L-lysine was 1:5 and 1:1.5 respectively (data not shown). According to these optimal ratios, 4-element complex and 5-element complex were prepared. The complexes were analyzed with 1% agarose gel electrophoresis to examine the retardation of DNA migration. A suitable complex gave a band trapped at the slot with no DNA migration (Fig 1).



**Fig 1.** 4-element complexes were prepared and the retardation of DNA was examined by 1% agarose gel electrophoresis. Each sample had 0.2  $\mu$ g DNA loaded into the sample well.

**A:** DNA retardation of different complexes using protamine as backbone

Lane 0: Plasmid DNA (pSV  $\beta$ -gal), 0.2  $\mu$ g Lane M: DNA marker,  $\lambda$  /HindIII

P.L-GV<sub>1</sub>: Add GV<sub>1</sub>/Protamine/HA20 to form complex with pSV  $\beta$ -gal DNA

P.L-GV<sub>2</sub>: Add GV<sub>2</sub>/Protamine/HA20 to form complex with pSV  $\beta$ -gal DNA

Lane 1: Plasmid DNA 0.2  $\mu$ g and GV1/Protamine/HA20 or GV2/Protamine/HA20 0.2  $\mu$ g(1:1)

Lane 2: Plasmid DNA 0.2  $\mu$ g and GV1/Protamine/HA20 or GV2/Protamine/HA200.4  $\mu$ g(1:2)

Lane 3: Plasmid DNA 0.2  $\mu$ g and GV1/Protamine/HA20 or GV2/Protamine/HA20 0.6  $\mu$ g(1:3)

Lane 4: Plasmid DNA 0.2  $\mu$ g and GV1/Protamine/HA20 or GV2/Protamine/HA20 0.8  $\mu$ g(1:4)

Lane 5: Plasmid DNA 1.0  $\mu$ g and GV1/Protamine/HA20 or GV2/Protamine/HA20 0.8  $\mu$ g(1:5)

**B:** DNA retardation of different complexes using poly-L-lysine as backbone

Lane 0: Plasmid DNA (pSV  $\beta$ -gal), 0.2  $\mu$ g Lane M: DNA marker,  $\lambda$  /HindIII

P.L-GV<sub>1</sub>: Add GV<sub>1</sub>/polylysine/HA20 to form complex with pSV  $\beta$ -gal DNA

P.L-GV<sub>2</sub>: Add GV<sub>2</sub>/polylysine/HA20 to form complex with pSV  $\beta$ -gal DNA

Lane 1: Plasmid DNA 0.2  $\mu$ g and GV<sub>1</sub>/polylysine/HA20 or GV<sub>2</sub>/polylysine/HA20 0.1  $\mu$ g (2:1)

Lane 2: Plasmid DNA 0.2  $\mu$ g and GV<sub>1</sub>/polylysine/HA20 or GV<sub>2</sub>/polylysine/HA20 0.13  $\mu$ g (1.5:1)

Lane 3: Plasmid DNA 0.2  $\mu$ g and GV<sub>1</sub>/polylysine/HA20 or GV<sub>2</sub>/polylysine/HA20 0.2  $\mu$ g (1:1)

Lane 4: Plasmid DNA 0.2  $\mu$ g and GV1/polylysine/HA20 or GV2/polylysine/HA20 0.4  $\mu$ g(1:2)

## Gene delivery system targeting VEGF receptors

### *Gene transfer in vitro to A375 cells and ABAE cells*

A375 cells were transfected with complexes GV1/HA20/poly-L-lysine/ $\beta$ -gal, GV2/HA20/poly-L-lysine/ $\beta$ -gal (Fig 2A), GV1/HA20/protamine/ $\beta$ -gal, GV2/HA20/protamine/ $\beta$ -gal and GV1/GV2/protamine/HA20/ $\beta$ -gal (Fig 2B) containing 0.2  $\mu$ g per ml medium. Though the gene expression level in cells transfected with complexes containing poly-L-lysine seemed slightly higher than that in cells transfected with complexes containing protamine, the difference is not really distinct. Using either protamine or poly-L-lysine as a backbone in gene delivery system, the gene transfer efficiency was similar in general for A375 cells (50% and 60% respectively).

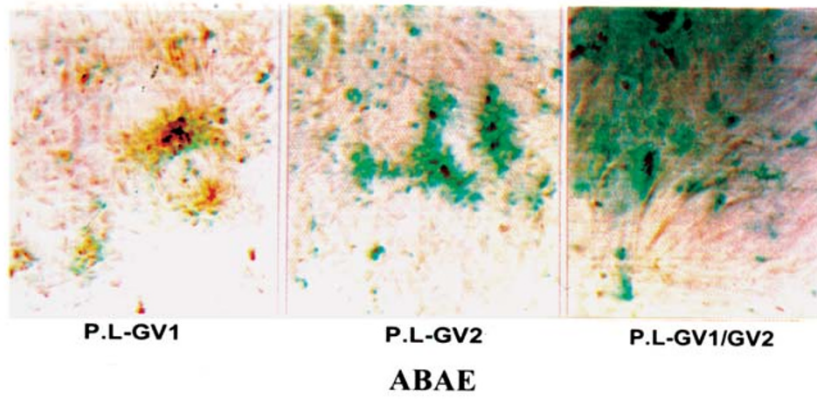
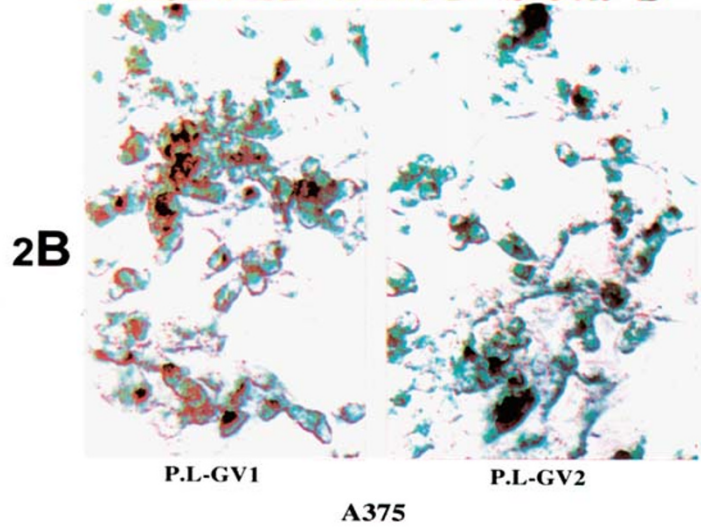
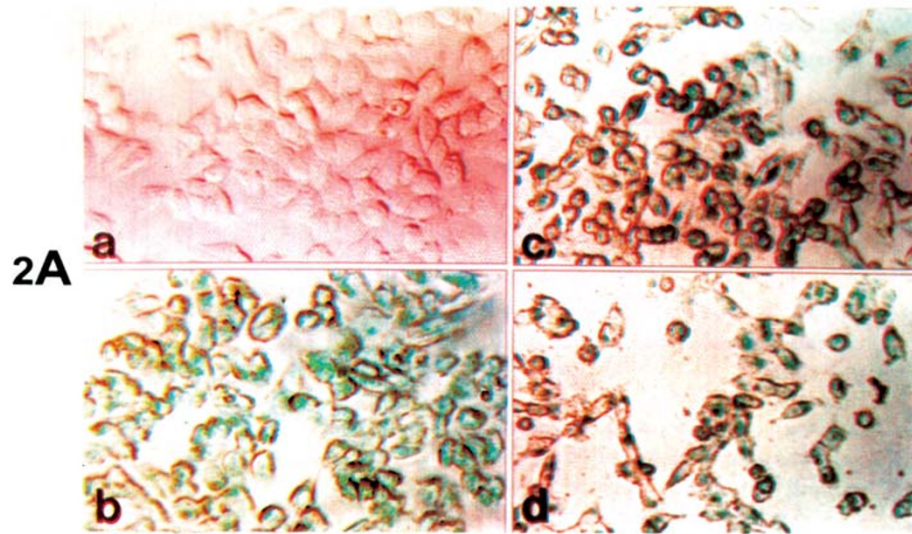
Using the same procedures, ABAE cells (Fig 3) were transfected with 4-element complex and 5-element complex with poly-L-lysine as a backbone containing 0.2  $\mu$ g DNA per ml medium.  $\beta$ gal expression level was about 70%. On the contrary,  $\beta$ -gal expression was at a low level (30%) provided that poly-L-lysine was replaced with protamine (data not shown). These results might be correlated to the expression level of VEGF receptors, as the binding of VEGF with its receptor could be inhibited by protamine. The effect of poly-L-lysine and protamine on VEGF receptors mediated gene delivery would be published elsewhere.

### *Competition inhibition test*

For competitive inhibition, various amount of GV2 at a range up to 5  $\mu$ g/ml was added to the A375 cells in a medium containing GV2/protamine/HA20/ $\beta$ -gal (0.2  $\mu$ g/ml). Cells were cultured and stained with X-gal to examine the competitive inhibition effect of GV2 on GV2 mediated gene transduction and to evaluate its

- ▷ **Fig 2.** Expression of  $\beta$ -galactosidase gene in A375 cells transfected with different gene delivery system containing 0.2  $\mu$ g plasmid DNA per ml medium.  
**2A:** A375 cells transfected with complex GV1/poly-L-lysine/HA20/ $\beta$ -gal (P.L-GV1, 400  $\times$ ) and GV2/poly-L-lysine/HA20/ $\beta$ -gal (P.L-GV2, 400  $\times$ ). The gene transfer efficiency was about 60%.  
**2B:** Gene transfer mediated by complexes containing protamine, the gene transfer efficiency was about 50%.  
(a) Human malignant melanoma A375 cells with PBS as control (400  $\times$ ).  
(b) A375 cells transfected with complex GV2/protamine/HA20/ $\beta$ -gal (400  $\times$ ).  
(c) A375 cells transfected with complex GV1/protamine/HA20/ $\beta$ -gal (400  $\times$ ).  
(d) A375 cells transfected with GV1/GV2/protamine/HA20/ $\beta$ -gal (400  $\times$ ).
- ▷ **Fig 3.** Expression of  $\beta$ -galactosidase gene in ABAE cells transfected with different gene delivery system containing 0.2  $\mu$ g plasmid DNA per ml medium. The gene transfer efficiency was about 70%.  
P.L-GV1: ABAE cells transfected with complex GV1/poly-L-lysine/HA20/ $\beta$ -gal (40  $\times$ ).  
P.L-GV2: ABAE cells transfected with complex GV2/poly-L-lysine/HA20/ $\beta$ -gal (40  $\times$ ).  
P.L-GV1/GV2: ABAE cells transfected with complex GV1/GV2/poly-L-lysine/HA20/ $\beta$ -gal (40  $\times$ ).

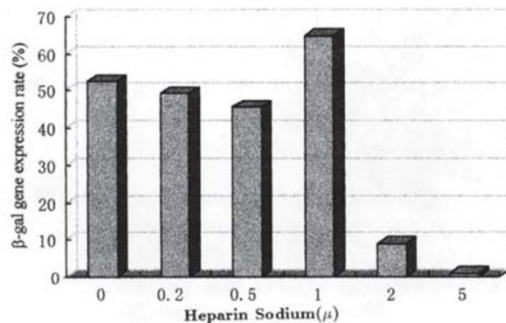




## Gene delivery system targeting VEGF receptors

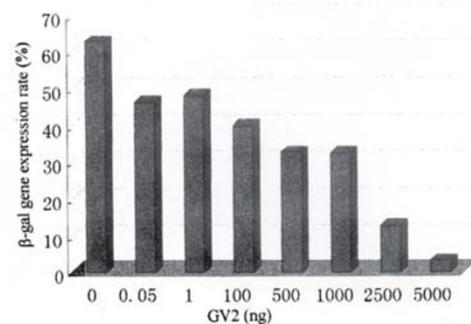
relative targetability. At a concentration of 50 pg/ml, gene transfer could be slightly inhibited (25%). Significant inhibition (50%) could be observed when the concentration increased to 500 ng/ml. At a concentration of 5  $\mu\text{g/ml}$  (1  $\mu\text{M}$ ), i.e. about 20 fold excess over the GV2 in GV2/protamine/HA20  $\beta$ -gal complex, gene transfer could be inhibited completely (more than 90%) (Fig 4). It is equivalent to the concentration of exogenous VEGF reported to competitively inhibit the toxicity of VEGF165-DT385 [14]. These results implicated that the gene transfer mediated by GV2 gene delivery system could possibly be a consequence of the interaction of oligopeptide GV2 and VEGF receptor expressed in A375 cells.

**Fig 4.** A375 cells were cultured in the medium containing GV2/protamine/HA20/  $\beta$ -gal complex (0.2  $\mu\text{g/ml}$ ) and various amount of GV2 at a range up to 5  $\mu\text{g/ml}$ . The  $\beta$ -gal gene expression rate was estimated by counting the blue granule staining in cells in three field of vision under light microscope. The gene transduction efficiency of the GV2 system was competitively inhibited by ligand oligopeptide GV2.



### *Effects of heparin sodium pretreatment*

Cells were incubated in medium with various amount of heparin sodium (0  $\mu\text{g/ml}$ , 0.2  $\mu\text{g/ml}$ , 0.5  $\mu\text{g/ml}$ , 1  $\mu\text{g/ml}$ , 2  $\mu\text{g/ml}$ , 5  $\mu\text{g/ml}$ ) at 37  $^{\circ}\text{C}$  for 30 minutes and were washed three times with 0.1 M PBS (pH 7.4) before adding fresh medium with 4-element complex GV2/protamine/HA20/ $\beta$ -gal (equivalent to 0.2  $\mu\text{g}$  DNA per ml medium). Cells were cultured and stained with X-gal at the 6th day to examine the influence of heparin sodium on the gene transfer efficiency. Pretreatment with heparin sodium at a concentration up to 0.5  $\mu\text{g/ml}$  could slightly inhibit the gene transfer efficiency. However, the gene transfer efficiency was slightly elevated at a concentration of 1mg/ml as compared to 0  $\mu\text{g/ml}$ , and the gene transfer efficiency is significantly inhibited when cells were pretreated

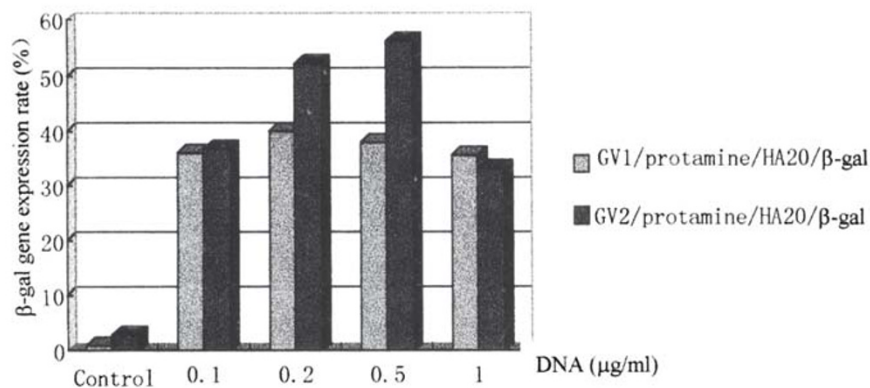


**Fig 5.**

A375 cells were pretreated with heparin sodium at a concentration range up to 5  $\mu\text{g/ml}$  before adding fresh medium with 4-element complex GV2/protamine/HA20/  $\beta$ -gal (equivalent to 0.2  $\mu\text{g}$  DNA per ml medium). The  $\beta$ -gal gene expression rate was estimated by counting the blue granule staining in cells in three field of vision under light microscope.



in medium with 2  $\mu\text{g/ml}$  heparin sodium was added (Fig 5). It had been reported that 1  $\mu\text{g/ml}$  heparin sodium could significantly enhance the binding capability of VEGF to its receptors[15]. Heparin has a dual effect of this particular receptor-mediated gene transfer system. It has been reported that heparin could enhance the binding of VEGF or VEGF like ligand to its receptor; meanwhile, it can neutralize the positive charges of protamine in the present system. Furthermore, the protamine in the complex has been slightly in excess, as the positive charges could possibly facilitate the interaction of the complex with cells which had negative charges on the surface. The slight enhancement effect of heparin at 1  $\mu\text{g/ml}$  observed in our experiments might possibly reflect the balanced dual effects of the enhancing activity of heparin on binding of VEGF to its receptor and the neutralizing reaction with polycationic polypeptide. When the concentration of heparin further increased, the binding capability of VEGF to its receptor would be reduced and the DNA could be dissociated from the polycationic polypeptide, thereby leading to a tremendous reduction of gene transfer efficiency.

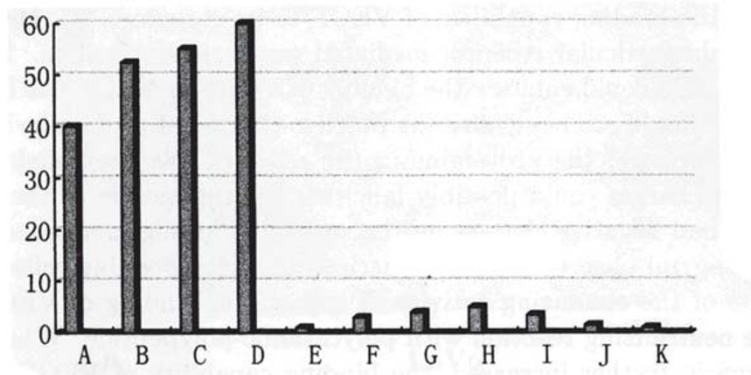


**Fig 6.** A375 cells were cultured in medium containing various dosage of composite polypeptide/DNA complex prepared according to optimal ratio of 5:1. The dosage of complex was represented by the amount of DNA content ( $\mu\text{g/ml}$ ) in the complex. Cells were fixed and stained. The  $\beta$ -gal gene expression rate was estimated by counting the blue granule staining in cells in three field of vision under light microscope. A375 cells transfected with GV1/protamine/ $\beta$ -gal and GV2/protamine/DNA dosage 3-element complex were as control.

#### *The influence of dosage of composite polypeptide/DNA complex on gene transfer efficiency*

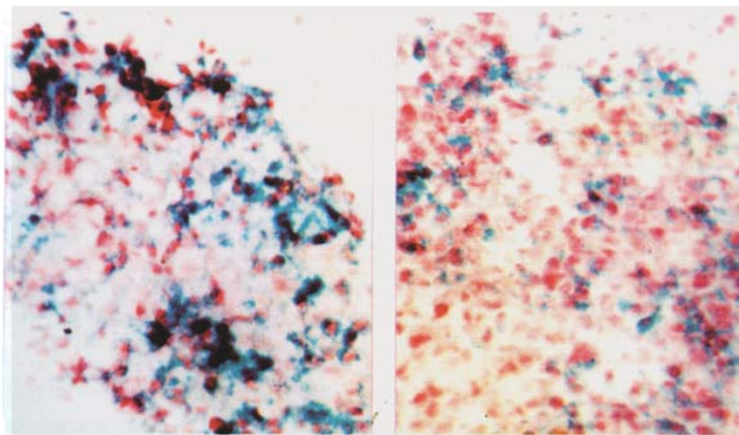
Cells were transfected with various amount 4-element complex GV2/protamine/HA20/ $\beta$ -gal DNA. The dosage was represented by the amount of DNA ( $\mu\text{g/ml}$ ) contained in the complex which was prepared according the optimal ratio presented in the previous section section. At a dosage containing 0.5  $\mu\text{g/ml}$  of DNA, the gene transfer

## Gene delivery system targeting VEGF receptors

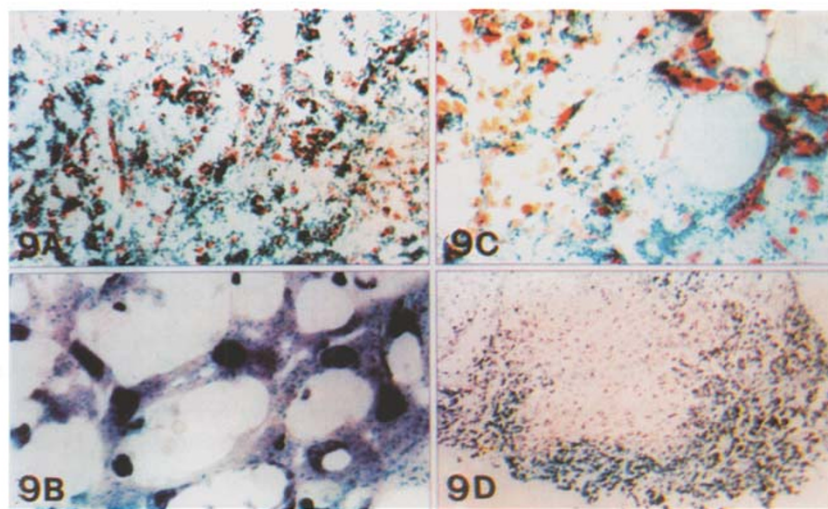
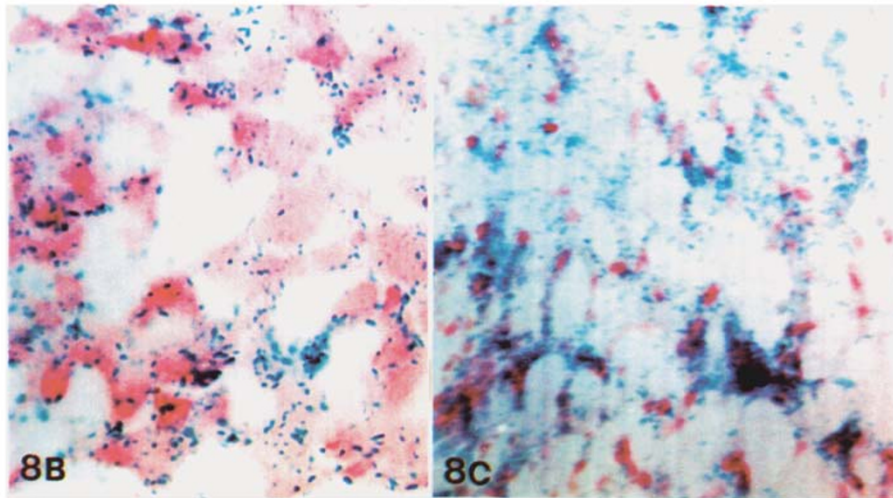


**Fig 7.** Various combination of the components of the GV1 Or GV2 system, PCP, GV1/ GV2-PCP and HA20/PCP, were used to form a complex with  $\beta$ -gal DNA. A375 cells were cultured in medium containing  $0.2 \mu\text{g}/\text{ml}$  different complex. The  $\beta$ -gal gene expression rate was estimated by counting the blue granule staining in cells in three field of vision under light microscope. A375 cells transfected with complex GV1/protamine/HA20 $\beta$ -gal (bar A), GV2/protamine/HA20/  $\beta$ -gal (bar B), GV1/poly-L-lysine/ HA20/  $\beta$  beta-gal (bar C), GV2/protamine/HA20/ $\beta$ -gal (bar D), GV1/protamine / $\beta$ -gal (bar E), GV2/ protamine/ $\beta$ -gal (bar F), GV1/poly-L-lysine/ $\beta$ -gal (bar G), GV2/poly-L-lysine/ $\beta$ -gal (bar H), HA20/ poly-L-lysine/ $\beta$ -gal (bar I), HA20/protamine/ $\beta$ -gal (bar J), protamine/  $\beta$ -gal (bar K). Results indicated that the transduction occurred by treatment with 2-element or 3-element complex was at a very low efficiency as compared with the highly efficient complete 4-element complex.

- ▷ **Fig 8.** Human colon cancer LOVO, malignant melanoma A375 and hepatoma xenograft transplanted subcutaneously in nude mice were used as animal model. GV1 or GV2 system was injected sutcutaneously into mice surrounding the tumor mass at a dose of complex containing  $0.2 \mu\text{g}$   $\beta$ -gal DNA per mouse. Frozen sections were counterstained with Nuclear Fast Red and examined under microscope
  - A:** Gene transfer to LOVO by GV1/protamine/HA20/ $\beta$ -gal (P-GV1,  $400\times$  and GV2/protamine/ HA20/ $\beta$ -gal (P-GV2,  $400 \times$ ).
  - B:** Gene transfer to A375 by GV1/protamine/HA20/ $\beta$ -gal ( $400 \times$ ).
  - C:** Gene transfer to hepatoma graft by GV2/protamine/ HA20/ $\beta$ -gal ( $400 \times$ ).
- ▷ **Fig 9.** GV2/protamine/HA20/ $\beta$ -gal ( $0.5 \mu\text{g}$  DNA/ $50\mu\text{l}$ ) was injected into portal vein of mice in which human hepatic cancer was transplanted in liver. Frozen sections were counterstained with Hematoxyline or Fast Nuclear Red and examined under microscope.
  - A:** Frozen section counterstained with Hematoxyline and showed hepatoma cells ( $400\times$ )
  - B, C:** Frozen section counterstained with Hematoxyline and showed vascular endothelial cells of tumor capillaries and small blood vessels ( $1000\times$ )
  - D:** Frozen section counterstained with Fast Nuclear Red and showed hepatoma cells in periheral infiltration area ( $40\times$ )



**8A** P-GV1 LoVo P-GV1



## Gene delivery system targeting VEGF receptors

efficiency of GV2 gene delivery system was dose-dependent; but the efficiency declined when complex with  $1\mu\text{g/ml}$  DNA was added. The decrease of gene transfer efficiency might be attributed the excess of protamine present in the complex, which could inhibit the binding of VEGF to its receptors at a given dosage[16]. The transfer efficiency of GV1 system gave the similar pattern though the decline at high dosage was not as prominent as GV2 (Fig 6). The differences in the extent of activities remain to be further studied.

### *The biological role of different moieties in GV2 gene delivery system*

To study the role of GV2 oligopeptide (LOP), HA20 oligopeptide (EROP) in the gene delivery system, in vitro gene transfer experiments were performed by utilizing 3-element complex (GV1/PCP/ $\beta$ -gal, GV2/PCP/ $\beta$ -gal and HA20/PCP  $\beta$ -gal), 2-element complex (PCP/ $\beta$ -gal) as compared with the complete 4-element system (GV1/PCP/HA20/ $\beta$ -gal and GV2/PCP/HA20/  $\beta$ -gal). As illustrated in Fig 7, 2-element complex and 3-element complex gave a very low efficiency of gene transduction. On the contrary, the complete 4-element system evidently revealed a highly efficient  $\beta$ -gal gene transduction. Therefore, it was implicated that all these elements, LOP, PCP and EROP, were essential to construct a full functional gene delivery system which is capable to target exogenous gene to cancer cells with high efficacy in gene transfer and its expression.

### *In vivo gene transduction to nude mice transplanted s.c. with human cancers*

Nude mice subcutaneously transplanted with human colon cancer LOVO (Fig 8A), human malignant melanoma A375 (Fig 8B), human hepatoma graft (Fig 8C) were injected s.c. with GV1 or GV2 4-element complex with a dose equivalent to  $0.2\mu\text{g}$  DNA per mouse.  $\beta$ -gal activity was observed in tumor vascular endothelial cells and cancer cells at the peripheral area of the tumor. It was correlated with the expression of VEGF receptors (the expression of VEGF receptors would be published elsewhere). These in vivo results did not show the difference between the  $\beta$ -gal activity in tumor cells and tumor endothelial cells on contrary to those results shown in in vitro experiments. The possible interpretation might be that the endogenous heparin or heparin-like proteins could cancel out the inhibitory effect of protamine on the binding of VEGF or related ligand to its receptors.

### *In vivo gene transduction mediated by GV2/protamine/HA20/ $\beta$ -gal 4-element complex to target genes into human tumors intrahepatically transplanted in nude mice*

$50\mu\text{l}$  GV2 4-element complex containing  $0.5\mu\text{g}$  pSV2  $\beta$ -gal DNA was injected into portal vein of mice in which a highly metastatic transplantable hepatoma was implanted in liver. High  $\beta$ -gal activity was observed in tumor cells (Fig 9A) and vascular



endothelial cells of tumor capillaries and small blood vessels (Fig 9B, C) particularly in area close to necrosis.  $\beta$ -gal expression was also observed in hepatoma cells in peripheral infiltration area (Fig 9D). However, only low  $\beta$ -gal activity was observed in endothelial cells of large blood vessels. No  $\beta$ -gal expression was observed in tumor necrotic area and normal liver cells.

#### *Delivery and expression of $\beta$ -gal gene in normal tissues*

$\beta$ -gal gene expression was examined at the same time in normal tissues of mice injected with the complex either subcutaneously or via portal vein. No  $\beta$ -gal activity was observed in heart, brain and lung. The surface of liver and spleen was stained with X-gal; however, no  $\beta$ -gal activity was observed under microscopic examination in frozen sections of these organs.  $\beta$ -gal expression was observed in individual renal tubule epithelial cells of kidney and in some glandular cells of stomach, small intestine and colon (data not shown).

## **DISCUSSION**

It had been reported that vascular endothelial growth factor (VEGF165) and a truncated diphtheria toxin molecule (DT385) could be linked together and the resulting conjugate (VEGF165-DT385) could inhibit human umbilical vein endothelial cell (HUVEC) proliferation while free toxin or a mixture of toxin polypeptide and VEGF did not affect endothelial cell proliferation. The selective cytotoxicity correlated with the appropriate receptor expression on the target cells[14]. To the best of our knowledge, there are no other articles reported previously about a gene delivery system mediated by VEGF receptors. In this paper, we described that GV1 or GV2 gene delivery system could transfer exogenous gene to cells with VEGF receptors expression in vitro and in vivo; while 2-element complexes and 3-element complexes could poorly transfer  $\beta$ -gal gene into A375 cells in vitro.

It also was demonstrated that the LOP (GV1, GV2), EROP (HA20) and PCP (poly-L-lysine or protamine) in gene delivery system were essential to display the full biological function of the composite vector system for gene delivery. Firstly, GV1 and GV2 were ligands for VEGF receptor and could target the DNA-polycation-ligand complex to cells by receptor binding and endocytosis. GV1 with 32 amino acids and GV2 with 36 amino acids were designed according to the putative binding region of VEGF to its receptors. They maintained the capability of binding VEGF receptors but they were smaller than VEGF165 with 165 amino acids. They would have less possibility in intramolecular conformational alteration as compared with using whole growth factor macromolecule or a viral envelope protein as moieties of the composite polypeptide vector system. They also could reduce the immune risk of the macromolecule. Secondly, HA20 (an endosome releasing domain from influenza virus haemagglutinin) enabled the endocytosed polypeptide/DNA complex to escape



## Gene delivery system targeting VEGF receptors

the lysosome fusion and subsequent degradation[17]. EROP might play an important role to keep the transduced gene intact, thereby increase its expression in cells. Lastly, the polycationic moiety such as poly-L-lysine or protamine was the backbone to interact with DNA by electrostatic binding to form a soluble complex.

However, the complicated mechanism of VEGF receptor mediated endocytosis remained to be further elucidated. The experiment data presented in this context would possibly provide some indirect, at least, evidence to support that the GV1 or GV2 system could transfer gene via VEGF receptor mediated mechanism. For instance, the oligopeptide ligand GV2 competitively inhibited the gene transfer efficiency of GV2/protamine/HA20/ $\beta$ -gal 4-element complex. In addition, heparin sodium at the concentration of 1  $\mu$ g/ml could enhance the gene transfer efficiency of GV2/protamine/HA20/ $\beta$ -gal 4-element complex. Furthermore, the results of  $\beta$ -gal gene transfer were well correlated to the presence of VEGF receptor revealed by cytochemical analysis (which would be published elsewhere). These data suggested that the gene delivery by GV1 or GV2 system could possibly be a consequence of interaction of the oligopeptide and VEGF receptors, especially GV2 gene delivery system.

Taken together, the present GV1 and GV2 system could target gene to tumor vascular endothelial cells. Since tumor growth is angiogenesis dependent, it seems possible that this system could potentially target exogenous genes to most solid tumors. However, there are some limitations in this system. For example, this system is not appropriate to be applied in the pregnant women or women with corpora luteum regeneration and the patients with wound or inflammation lesion, because angiogenesis is also important under these physiological or pathological circumstances[18]. Studies are under way in order to further improve the VEGF receptors-mediated gene delivery system for future clinic trials of cancer gene therapy.

## ACKNOWLEDGEMENTS

The present study has been granted by Biotechnology Project, National High Technology Program (Project No. Z-20-01-01). We thank Professor SHENG Minli and JIANG Yamei in Institute of Medical Radiology, Shanghai Medical University for their kind gift of ABAE cells.

## REFERENCES

- [1] George Y Wu, Catherine H Wu. Receptor-mediated in vitro gene transformation by a soluble DNA carrier system. *J Biol Chem* 1987; **262**(10):4429-32.
- [2] Ernst Wagner, Martin Zenke, Matt Cotton et al. Transferrin-polycation conjugates as carriers for DNA uptake into cells. *Proc Natl Acad Sci USA* 1990; **87**:3410-4.
- [3] Tian Peikun, Ren Shengjun, Ren Changchun et al. A Novel Receptor-targeted Gene Delivery System for cancer gene therapy, *Science in China (Series C)*, accepted.
- [4] Ulla Wienhues, Keiichi Hosokawa. Arnd Hoveler et al. A novel method for transfection and expression of reconstituted DNA-protein complexes in eukaryotic cells, *DNA*. 1987; **6**(1):81-9.
- [5] Judah Folkman, Yuen Shing. Angiogenesis. *J Biol Chem* 1992; **267**(16):10931-34.
- [6] Napoleone Ferrara. Vascular endothelial growth factor. *Trends Cardiovasc Med* 1993; **3**:244-50.

- [7] Erika Hatva, Arta Kaipainen, Panu Mentula et al. Expression of endothelial cell specific receptor tyrosine kinases and growth factors in human brain tumors, *American Journal of Pathology*. 1995; **146(2)**: 368-78.
- [8] Kunio Suzuki, Norio Hayashi, Yasuhide Miyamoto et al. Expression of vascular permeability factor/vascular endothelial growth factor in human hepatocellular carcinoma, *Cancer Res* 1996; **56**:3004-9.
- [9] Christine A Boocock, D Stepjen Charnock-Jones, Andrew M Sharkey et al. Expression of vascular endothelial growth factor and its receptors flt and KDR in ovarian carcinoma. *J Nat Cancer Inst* 1995; **87**:506-16.
- [10] Bo Liu, Helena M Earl, Dilair Baban et al. Melanoma cell lines express VEGF receptor KDR and respond to exogenously added VEGF. *Biochem Biophys Res Commun* 1995; **217(3)**:721-7.
- [11] Adrian L Harris, Huatang zhang, Amir Moghaddam et al. Breast cancer angiogenesis-new approaches to therapy via antiangiogenesis, hypoxic activated drugs and vascular targeting, *Breast Cancer Research and Treatment*. 1996; **38**:97-108.
- [12] Karl H Plate, Georg Breier, Herbert A Weich et al. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo *Nature*, 1992; **359**:845-8.
- [13] Mingli Sheng et al. Culture of Vascular Endothelial cell 1987; **14(1)**:71-3.
- [14] S. Ramakrishnan, TA Olson, VL Bautch et al. Vascular endothelial growth factor-toxin conjugate specifically inhibits KDR/flk-1 positive endothelial cell proliferation in vitro and angiogenesis in vivo. *Cancer Res* 1996; **56**:1324-30.
- [15] Hela Gitary-Goren, Shay Soker, Israel Vlodavsky et al. The binding of vascular endothelial growth factor to its receptors is dependent on cell surface associated heparin-like molecules. *J Biol Chem* 1992; **267(9)**:6093-8.
- [16] G Neufeld, D Gospodarowicz. Protamine sulfate inhibits mitogenic activities of the extracellular matrix and fibroblast growth factor, but potentiates that of epidermal growth factor. *J Cell Phys* 1987; **132**: 287-94
- [17] Masayuki Murata, Satoshi Kagiwada, Sho Takahashi et al. Membrane Fusion induced by mutual interaction of the two charge-reversed amphiphilic peptides at neutral pH. *J Biol Chem* 1991; **266(22)**: 14353-8.
- [18] Judah Folkman. What is the evidence that tumors are angiogenesis dependent *J Nat Cancer Inst* 1990; **88**:4-6.