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QUARTERLY

Construction of a bicistronic proangiogenic expression vector and its application in experimental angiogenesis *in vivo*

Maciej Małecki*, Małgorzata Przybyszewska and Przemysław Janik✉

Cancer Center, Department of Cell Biology, W. Roentgena 5, 02-781 Warszawa, Poland

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Manipulation of angiogenesis *in vivo* is an example of successful gene therapy strategies. Overexpression of angiogenic genes like VEGF, FGF or PDGF causes new vessel formation and improves the clinical state of patients. Gene therapy is a very promising procedure but requires large amounts of pharmaceutical-grade plasmid DNA. In this regard we have constructed a bicistronic plasmid DNA vector encoding two proangiogenic factors, VEGF165 and FGF-2. The construct (pVIF) contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV) which permits both genes to be translated from a single bicistronic mRNA. The IRES sequence allows for a high efficiency of gene expression *in vivo*. The pVIF vector was characterized *in vitro* and *in vivo*. *In vivo* angiogenesis studies showed that the bicistronic vector encoding two proangiogenic factors induces the formation of new vessels significantly more than pVEGF165 or pFGF-2 alone. In our opinion the combined proangiogenic approach with VEGF165 and FGF-2 is more powerful and efficient than single gene therapy. We also postulate that IRES sequence can serve as a useful device improving efficiency of gene therapy.

Angiogenesis is a multistep process involved in many physiological and pathological phenomena (Battegay, 1995; Szala & Radzikowski, 1997). The formation of new vessels is postulated to be a crucial point for tumorigenesis and metastasis, whereas deterioration of the arterial system is one of the reasons of ischemic diseases (Battegay, 1995; Szala & Radzikowski, 1997). Therefore, manipulation of angiogenesis *in vivo* may be a successful gene therapy strategy (Zhang & Harris, 1998; Hayes, 1999; Harjai *et al.*, 2002). In the last decade significant development of angiogenic gene therapy methods has been observed (Isner & Asahara, 1998; Harjai *et al.*, 2002). Many hopes are linked with application

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✉Corresponding author: tel/fax: (48 22) 644 9184, e-mail: pjanik@coi.waw.pl

Abbreviations: ECMV, encephalomyocarditis virus; FGF, fibroblast growth factor; HUVEC, human umbilical vein endothelial cells; IRES, internal ribosome entry site; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor.

of various expression vectors encoding angiogenic factors for the treatment of heart or hind limb ischemia (Isner & Asahara, 1998; Dulak *et al.*, 1999; Harjai *et al.*, 2002). Overexpression of angiogenic genes, for example VEGF, PDGF or FGF can cause the formation of new vessels and improve the clinical state of patients (Isner *et al.*, 1996a; 1996b; Isner & Asahara, 1998). In our previous study we described promising results of *in vitro* and *in vivo* experiments using pHVEGF165 vector (Małecki *et al.*, 2001). The clinical state of patients suffering from serious heart ischemia improved after intracardiac injection of our plasmid encoding VEGF165 (Kołsut *et al.*, 2003). Since VEGF as well as FGF belong to the family of proangiogenic growth factors a vector encoding two proangiogenic proteins would have an advantage over two or three single gene plasmids. In the present work we have prepared a new construct – a bicistronic DNA vector encoding two angiogenic factors, namely VEGF165 and FGF-2. It could be expected that combination of VEGF165 and FGF-2 would have higher proangiogenic efficiency than monocistronic plasmids. The new construct (pVIF) contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV). This sequence permits both genes of interest (VEGF165 and FGF-2) to be translated separately from a single bicistronic mRNA. Additional advantages of bicistronic vectors are lower cost of preparation and lower level of endotoxins in the single bicistronic vector preparation than in two monocistronic plasmids. We also expected that combined gene therapy with two angiogenic factors would induce more new vessels *in vivo* than could be obtained with monocistronic plasmid.

MATERIALS AND METHODS

Construction and preparation of bicistronic pVIF vector. The structure of the pSEC expression vector is shown in Fig. 1. The cloning procedure begun from the cloning of two expression vectors, pFGF-2 and pVEGF/IRES, encoding FGF-2 and VEGF165/IRES, respectively. Amplified fragments were inserted into pSEC expression vector (Invitrogen, Holland). The pSEC expression vector (Fig. 1) was amplified in *Escherichia coli* DH5 strain growing on LB medium (Sigma, U.S.A.) containing ampicillin (100 μg/ml). The plasmid was isolated with the EndoFree Plasmid Mega Kit (QIAGEN, Germany). Purity of the empty plasmid was confirmed spectrophotometrically and by agarose gel electrophoresis. The isolated plasmid was then digested with appropriate restriction enzymes.

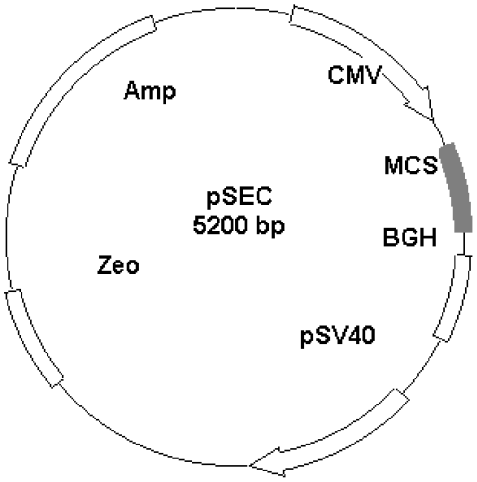

Figure 1. A schematic map of pSEC vector.

The functional elements of the vector are as follows: CMV, human cytomegalovirus immediate-early promoter/enhancer; MCS, multiple cloning site; BGH, bovine growth hormone polyadenylation signal; pSV40, early promoter and origin; Zeo, Zeocin resistance gene; Amp, ampicillin resistance gene. The bicistronic expression cassette was cloned into the MCS site.

A) Construction of pSEC/FGF-2 expression vector. A fragment of DNA encoding FGF-2 was amplified using cDNA from HUVEC (total cDNA of HUVEC was a gift of Dr. Józkowicz from Collegium Medicum, Kraków, Poland). PCR amplification of cDNA was performed using specific primers for FGF-2 carrying *Eco*RI and *Xho*I restriction

sites (Table 1). The product was digested with *Eco*RI and *Xho*I, separated by electrophoresis on 1.2% agarose gel with ethidium bromide (0.5 μg/ml) and isolated using Qiagen Gel Extraction Kit (QIAGEN, Germany). The obtained product was then ligated with *Eco*RI- and *Xho*I-digested pSEC vector for 16 h at 16°C in the presence of T4 ligase (Amershem, U.K.). The ligation mixture was transferred into a competent *E. coli*. The bacterial culture was incubated on LB agar followed by LB medium supplemented with ampicillin. After incubation individual clones were isolated with QIAprep Spin Miniprep Kit (QIAGEN, Germany) and analysed by digestion with restrictases. Finally, the pSEC/FGF-2 vector was sequenced.

Table 1. Parameters of PCR.

The table shows oligonucleotide (primer) sequences, melting temperature of duplex DNA and oligonucleotides and products size. F, forward primer; R, reverse primer; bp, base pair.

Gene	Primer sequence	Annealing temperature (^{O}C)	Product size (bp)
VEGF165	F:5'AAGGATCCGCACCCATG GCAGAAGGAGGAGG-3' R:5'TTGAATTCCCGAAACGC TGAGGGAGGCT-3'	63.8	549
IRES	F:5'AAGAATTCCAATTCCGC CCCTCTCCCT - 3' R:5'AAGCGGCCGCTTGTGGC AAGCTTATCATC - 3'	57.7	601
FGF-2	F^1:5'ATGAATTCGCCAGCATT GCCCGAGGAT - 3' R:5'TTCTCGAGATTCAGCTC TTAGCAGACAT - 3' F^2:5'AAGCGGCCGCACCATG GAGACAGACACA3'	54.7 (F^1+R) 57.0 (F^2+R)	460 (F^1+R) 596 (F^2+R)

B) Construction of pSEC/VEGF/IRES expression vector. Construction of the pSEC/VEGF165 expression vector was described previously (Małecki *et al.*, 2001). The plasmid pSEC/VEGF165/IRES was obtained after amplification by PCR (Table 1) and subcloning of the *Eco*RI/IRES/*Not*I fragment from the pMIG retroviral vector containing the sequence of the internal entry site (IRES) into the plasmid pSEC/VEGF cleaved by *Eco*RI and N*ot*I (pMIG vector was a gift of Dr. Skórski from Temple University, Philadelphia, U.S.A.). The IRES sequence is derived from encephalomyocarditis virus (ECMV) and permits the translation of two open reading frames from one messenger RNA. The obtained vector was isolated and analysed as described above.

C) Construction of bicistronic pSEC/VEGF165/IRES/FGF-2 expression vector. The *Not*I-ATG/Igk/FGF-2/*Xho*I sequence from pSEC/FGF-2 vector was amplified by PCR (F^1+R; Table 1) and cloned into pSEC/VEGF165/IRES vector digested with *Not*I and *Xho*I. The obtained vector was analysed as described previously. The structure of the cloned bicistronic expression cassette

containing the VEGF165 gene followed by IRES sequence and FGF-2 gene is shown in Fig. 2.

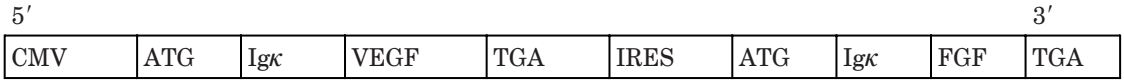

Figure 2. The bicistronic expression cassette cloned into the pSEC vector.

The elements of the cassette are as follows: CMV, human cytomegalovirus immediate-early promoter/enhancer; ATG, initiation codon; Ig, leader sequence; VEGF, vascular endothelial growth factor 165 gene; TGA, termination codon; IRES, internal ribosome entry site; FGF, basic fibroblast growth factor (FGF-2).

PCR analysis of the pVIF bicistronic vector. The pVIF bicistronic vector as well as pSEC, pVEGF and pFGF were analysed by standard PCR. Amplification of cDNA cloned into pSEC vector was performed using specific primers for VEGF165, IRES and FGF-2. The sequences of the primers and the reaction conditions are shown in Table 1. The products were separated by electrophoresis on 1.2% agarose gel with ethidium bromide (0.5 μg/ml).

Cell culture and transfection procedure. The mouse sarcoma cell line L1 was used in the experiments. Cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum and 0.1 mg/ml penicillin + streptomycin at 37°C in a humidified atmosphere of 5% CO_2. The pVIF vector was transfected into L1 cells by lipotransfection according to the manufacturer's protocol (Przybyszewska *et al.*, 1998). Zeocin resistant clones of the L1 transfected cells were analysed directly for VEGF and FGF overexpression.

Western blot assay. VEGF165 and FGF-2 proteins were detected in the conditioned medium of pVIF-transfected L1 cells by Western blotting with the use of anti-VEGF (Santa Cruz Biotechnology, Inc., sc-152) and anti-FGF (Santa Cruz Biotechnology, Inc., sc-1390) antibodies, respectively. The conditioned medium was 20 times concentrated using 10 kDa cut off centrifugal filter devices (Amicon, U.S.A.).

In vivo *angiogenesis assay.* Adult inbred female BALB/c mice (9–11 weeks old) received six intradermal injections of 10 μg of naked pVIF, pVEGF and pFGF-2 vectors. Three and thirteen days later, the mice were sacrificed, and new blood vessel formation on the inner surface of the skin was counted as described by Sidky and Auerbach (1975).

RESULTS

Cloning and validation of VEGF and FGF cDNAs

The structure of the bicistronic expression cassette cloned into the pSEC vector is shown in Fig. 2. The obtained pVIF vector was amplified in *E. coli* DH5. The plasmid was isolated with EndoFree Plasmid Mega Kit and its quality was confirmed spectrophotometrically and electrophoretically. Restriction digestion mapping and DNA sequencing of this vector confirmed the presence of VEGF165 and FGF-2 cDNAs. Restriction digestion results of the pVIF vector are shown in Fig. 3. The presence of VEGF165, IRES and FGF-2 inserts in the pVIF vector was also confirmed by PCR analysis (Fig. 4).

Western blotting study

To determine the expression/secretion of VEGF165 and FGF-2 *in vitro*, mouse sarcoma cells (L1) were transfected with pVIF. Western blot analysis showed a high level of VEGF and FGF proteins in the conditioned medium

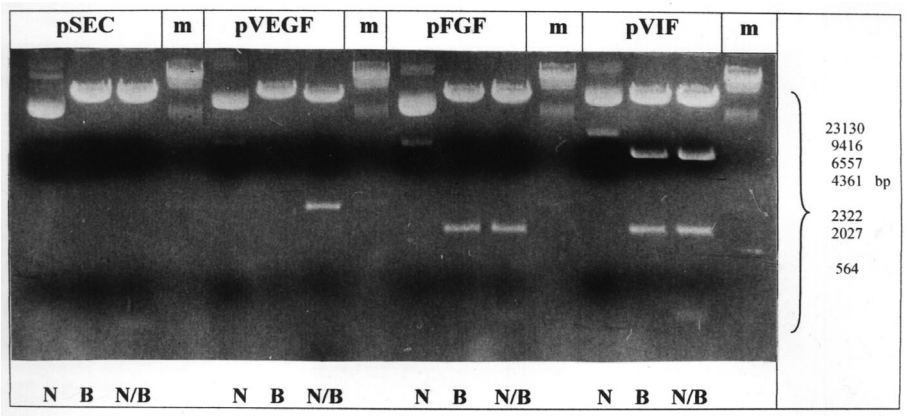

Figure 3. Restriction digestion mapping of pSEC, pVEGF, pFGF and pVIF vectors confirmed the presence of appropriate cDNAs.

The studied vectors were digested with *Bam*HI (B) and a combination of *Bam*HI and *Xho*I (N/B). N, non digested vector; m, size marker (λ/*Hin*dIII); bp, base pairs. The size of the digestion fragments are as follows (bp): pSEC: N, 5200, B, 5200, N/B, 5147 and 53; pVEGF: N, 5723, B, 5723, N/B, 5147 and 576; pFGF: N, 5621, B, 5216 and 405, N/B, 5144, 405 and 72; pVIF: N, 6960, B, 5245, 1310 and 405, N/B, 5173, 1310, 405 and 72 bp.

of the pVIF L1 transfected cells. As shown by quantitative analysis (1D Image Analysis, Kodak) of the VEGF and FGF bands, the pVIF transfectants revealed an about 25% higher level of VEGF than FGF. The results are shown in Fig. 5A–C.

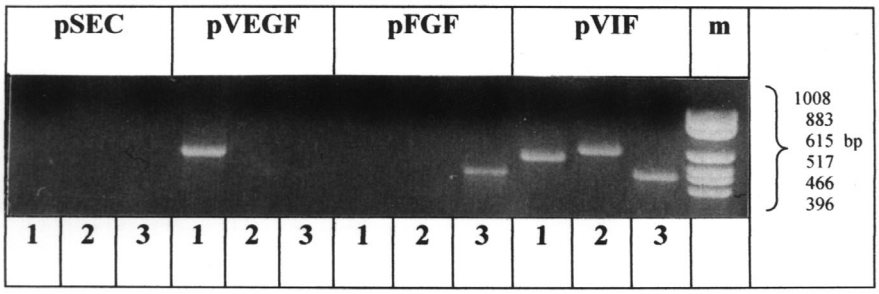

Figure 4. PCR analysis of the bicistronic pVIF vector.

The pVIF vector was analysed by standard PCR method. Presence of the VEGF165, IRES and FGF-2 inserts was confirmed by amplification of the cDNA according to data shown in Table 1. The size of the cDNAs is shown in Fig. 1, VEGF165 (549 bp); 2, IRES (601 bp); 3, FGF-2 (460 bp); m, size marker (pK03/*Hin*fI).

In vivo angiogenesis assay

To determine the effect of the bicistronic proangiogenic pVIF vector on the formation of new vessels *in vivo*, mice were intradermally injected with pVIF. As seen in Table 2 the formation of new vessels after intradermal injection of the naked proangiogenic vectors increased significantly compared with the injection of a plasmid without an angiogenic gene (empty plasmid). The bicistronic pVIF vector induced generation of new vessels twice that induced by monocistronic vectors, pVEGF or pFGF. These results confirm the ability of the pVIF vector to produce two angiogenic proteins *in vivo*. The IRES sequence enables a high efficiency of expression of both genes.

DISCUSSION

Angiogenesis — formation of new blood vessels — occurs in physiological and pathological

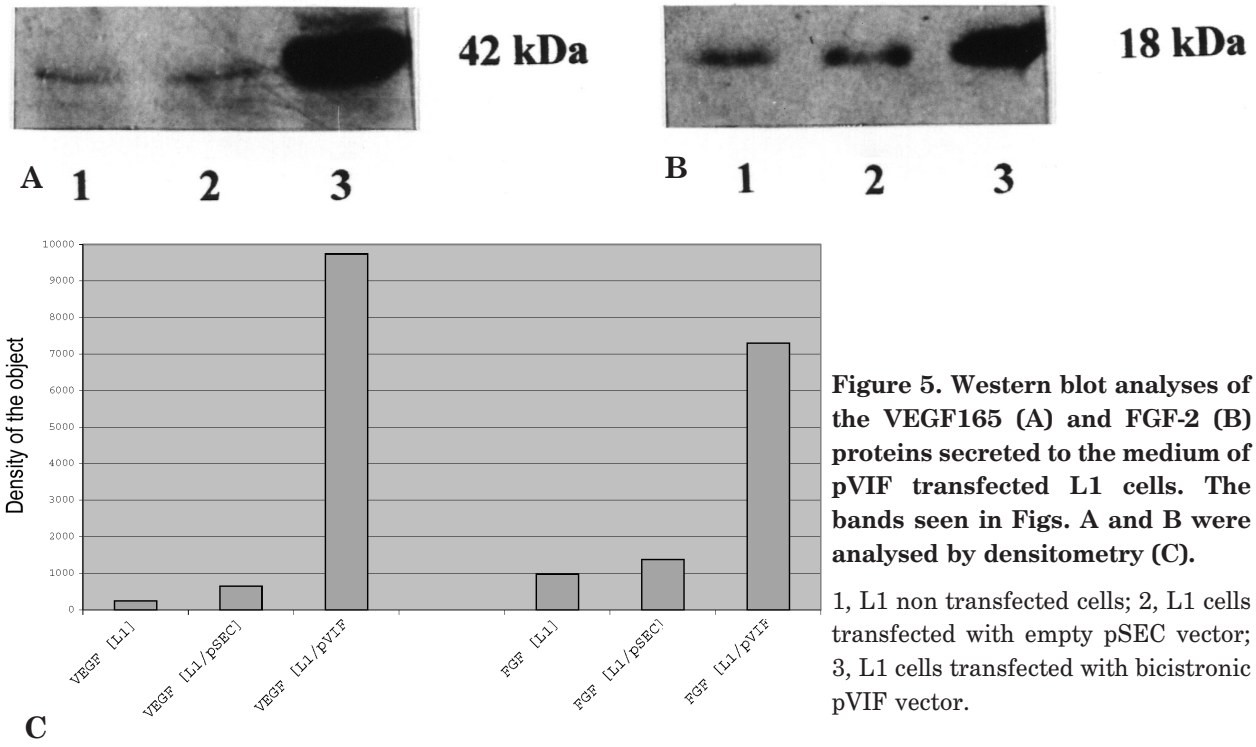

Figure 5. Western blot analyses of the VEGF165 (A) and FGF-2 (B) proteins secreted to the medium of pVIF transfected L1 cells. The bands seen in Figs. A and B were analysed by densitometry (C).

1, L1 non transfected cells; 2, L1 cells transfected with empty pSEC vector; 3, L1 cells transfected with bicistronic pVIF vector.

states. It is a multistep process that plays a crucial role in the development of vascular supply in normal tissue, e.g. in reproduction and wound healing. Angiogenesis is also involved in many ischemic diseases like heart disease, peripheral vascular disease, rheumatoid arthritis, and tumor growth and metastasis (Battegay, 1995; Szala & Radzikowski, 1997). Manipulation of angiogenesis is seen as a promising strategy for treatment of many diseases such as ischemic heart and peripheral vascular disease (Isner & Asahara, 1998; Harjai *et al.*, 2002; Małecki & Janik, 2002; Kołsut *et al.*, 2003). A multitude of angiogenic molecules have been well characterized (Battegay, 1995; Małecki & Janik, 1999). Many researches have found that transfer of angiogenic genes, e.g. VEGF or FGF, into ischemic tissues caused the formation of new vessels and improved the clinical state of patients (Isner *et al.*, 1996a; 1996b; Garcia-Martinez *et al.*, 1999; Harjai *et al.*, 2002). Most of the current gene therapy approaches are compromised by the inability to deliver

Table 2. The number of blood vessels found in animals injected intradermally with plasmid DNA encoding angiogenic factors.

Empty pSEC vector and 0.9% NaCl served as control. Results represent the mean ±S.D.

Day of measurement	0.9 % NaCl	pSEC	Angiogenic DNA vectors		
			pVEGF	pFGF	**pVIF**
3	4.5 ± 1.8 n = 18	7.90 ± 2.91 n = 15	14.50 ± 2.26 n = 14	12.13 ± 1.96 n = 15	21.33 ± 3.77 n = 15
13	-	3.90 ± 1.29 n = 10	18.20 ± 4.5 n = 15	7.86 ± 1.51 n = 14	26.10 ± 8.60 n = 17

genes efficiently to obtain sustained expression. One of the methods used is direct injection of naked plasmid DNA into muscles. It is a very efficient procedure but requires large amounts of pharmaceutical-grade plasmid DNA. We have constructed a bicistronic plasmid DNA vector to induce angiogenesis *in vivo via* high expression of two proangiogenic factors, namely VEGF165 and FGF-2. The pVIF vector was tested *in vitro* and *in vivo*. As seen in Figs. 3–4 digestion mapping and PCR analysis confirmed the presence of VEGF165 and FGF-2 cDNAs. Western blotting analysis (Fig. 5) showed that secretion of VEGF165 and FGF-2 proteins to the conditioned medium of the pVIF transfected L1cells was increased significantly comparing with non transfected cells. All cells positive for VEGF also expressed FGF. The IRES sequence permits both genes of interest — VEGF165 and FGF-2 — to be translated efficiently from a single bicistronic mRNA (Wiznerowicz *et al.*, 1998; Martinez-Salas, 1999; Vagner *et al.*, 2001). It is worth noticing that expression of FGF-2 was lower than VEGF165. These observations are consistent with those obtained in other studies. Mizuguchi *et al.* (2000) described that IRES-dependent second gene expression can be lower than of the first gene in a bicistronic vector. These findings were also confirmed by Kapturczak *et al.* (2002) who showed similar differences in the degree of expression levels for studied genes. To determine the effect of the bicistronic pVIF vector on angiogenesis *in vivo* mice were implanted intradermally with naked plasmid DNA and the formation of new vessels was monitored. Our studies demonstrate convincingly the increased number of newly formed vessels in animals injected with pVIF. The proposed system is based upon studies where combined expression of angiogenic cytokines has been used to achieve efficient angiogenesis *in vivo*. Ibukiyama showed that both exogenous VEGF and FGF can significantly promote collateral vessel development (Ibukiyama, 1996). It seems clear that combined angiogenic therapy can be an effective strategy for the treatment of ischemic disease. Similar to other researchers we postulate that IRES sequence can serve as a useful biotechnological tool improving the efficiency of gene therapy (Gurtu *et al.*, 1996; Attal *et al.*, 1999; Mizuguchi *et al.*, 2000; de Felipe, 2002; Singh *et al.*, 2002). In our opinion this sequence may be also helpful in angiogenic gene therapy. As we show here, the combined use of VEGF165 and FGF-2 is more powerful and efficient than single gene therapy. Additionally, the pVIF vector permits a lower cost of preparation and high level of purity. The content of endotoxins in the final pVIF preparations is almost always two times lower than in the mixture of two vectors (pVEGF and pFGF) isolated separately.

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