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(54) Title: EXPRESSION BOX, TWO CISTRON PLASMID VECTOR, PHARMACEUTICAL AND THEIR USE IN ANGIOGENIC GENE THERAPY

(57) Abstract: An expression box, two-cistron plasmid vector, pharmaceutical composition and their application in angiogenic gene therapy facilitate an increase in the secretion rate of expressed angiogenic factors, and the presented vector induces the formation of mature and stabilised blood vessels *in vivo*.



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EXPRESSION BOX, TWO-CISTRON PLASMID VECTOR, PHARMACEUTICAL
AND THEIR USE IN ANGIOGENIC GENE THERAPY

FIELD OF THE INVENTION

5 The present invention relates to an expression box, two-cistron plasmid vector, pharmaceutical composition and their application in angiogenic gene therapy.

BACKGROUND OF THE INVENTION

The therapeutic application of angiogenic growth factors was described for the first time by Folkman et al. (Folkman, N Engl J Med, 285:1182-1186 (1971)). Later studies have confirmed the utility of recombinant angiogenic growth factors, such as the fibroblast growth factor family (FGF) (Yanagisawa-Miwa, et al., Science, 257:1401-1403 (1992) i
10 Baffour, et al., J Vasc Surg, 16:181-91 (1992)), endothelial cell growth factor (ECGF)(Pu, et al., J Surg Res, 54:575-83 (1993)), as well as the vascular endothelial growth factor (VEGF) in the stimulation of development and maturation of new blood vessels in an
15 animal model of ischemia, particularly in cardiac muscle (Takeshita, et al., Circulation, 90:228-234 (1994) and Takeshita, et al., J Clin Invest, 93:662-70 (1994)). In the mentioned studies, recombinant proteins were applied intramuscularly. The significant limitation of this type of therapy are the difficulties in maintaining the correct concentration of growth factors at the treatment site.

20 An alternative method for recombinant protein treatment is the application of gene therapy, which consists of the insertion of DNA coding the factors in question into the affected site. Many attempts have been made to utilise sequences coding angiogenic growth factors in therapy.

Patent application WO 97/14307 pertains to a method of treatment of ischemia in a
25 mammal, which consists of injecting into the said tissue of an effective quantity of nucleic acid capable of the expression of the angiogenic protein. The method of the present invention may be applied in the treatment of any ischemic tissue, that is a tissue deficient in blood supply as a result of ischemia. Examples of these tissue are muscles (including cardiac muscle), the brain, kidneys and lungs. A plasmid has been described containing the
30 cDNA of human VEGF-165 under control of the CMV promoter. On page 9, paragraph 3,

it has been stated that “a beneficial angiogenic protein contains a secretory signal sequence which facilitates the secretion of the protein. Also of benefit are angiogenic proteins possessing native signal sequences, i.e. VEGF. Angiogenic proteins without a native signal sequence, i.e. bFGF, may be modified using known methods, so that they may possess such a sequence (...)” The problem posed in this way does not take into account the possibility of replacing the native secretory sequence of VEGF-165 with another known sequence, i.e. in order to improve secretion, and suggests that the most appropriate sequences are those natively appearing in the sequence of a given protein.

Animal tests were described (rabbits and calves), which presented qualitative and quantitative evidence of the attainment of the desirable physiological effect. There are no data reporting human therapy.

Patent application WO 97/12519 pertains to a method of the re-endothelisation damaged blood vessels with gene therapy. A plasmid containing the cDNA of human VEGF-165 under the control of the CMV promoter was applied. The expression box came from phVEGF-165 described in WO 97/14307 (above). The description also contains the same statements regarding the secretory sequence.

Animal tests were described (rabbits and calves), which presented qualitative and quantitative evidence of the attainment of the desirable physiological effect. There are no data reporting human therapy.

Patent application WO 00/24412 pertains to products and methods of prevention of the constriction or re-constriction of vessels using the VEGF-C and/or VEGF-D genes and proteins.

On page 7 of the description (lines 8-19) the authors demonstrated possible changes to the VEGF-C sequence used, and at the same time they underlined the importance of the secretory sequence in the proper secretion of the planned proteins. They suggested the application of a secretory sequence from VEGF-C along with the described derivatives of this protein. It was also suggested to change the native secretory sequence for another secretory sequence.

Patent application WO 01/34208 pertains to the treatment of patients with circulatory illness, including heart disease and peripheral circulatory system disorders. The methods

under the invention include the *in vivo* administration of genes coding angiogenic peptides or proteins into heart muscle or peripheral tissue affected with poor blood flow, through the introduction of a vector containing a gene into blood vessels supplying the heart or into the ischemic tissue. Example 7 reveals the results of an experiment whose aim was to increase the level of the angiogenic protein FGF-2 possessing no native expression sequence. The desired effect (an increase in the stimulation of angiogenesis) was achieved by concatenating the secretory sequence of FGF-4. It was suggested that the sequence added should stem from a protein expressed in cardiac muscle cells. It was also suggested that it may also be beneficial to utilise the FGF-4 and VEGF-145 genes in parallel, which according to the authors may lead to a synergistic effect. No evidence was given, however, to support this conjecture. Only animal tests were described. There are no data describing the therapy in humans.

THE GOAL OF THE INVENTION

In the light of the current state of knowledge there still remains a need for therapeutic agents which could be used in gene therapy of circulatory illness in humans. Problems which need to be solved involve the efficacy of the administered medicines, for example through increasing the level of secretion of the expressed angiogenic factors. Other problems in need of solution are the quality and durability of the induced vessels, which should reach full maturity in order to improve the therapeutic effect. It would also be beneficial to limit the potential toxicity of injection mixtures containing the therapeutic DNA, as well as to lower their cost.

The problems indicated were solved in the present invention.

SUMMARY OF THE INVENTION

The present invention provides an expression box consisting of a CMV promoter, and the polyadenylation signal sequence SV40, connected operationally with a gene complex comprised of:

- a) a sequence coding a hybrid secretory protein derived from immunoglobulin connected to a VEGF₁₆₅ sequence lacking a native secretory sequence,
- b) a sequence coding a hybrid secretory protein derived from immunoglobulin connected to a bFGF,

c) an IRES sequence located in between the mentioned sequences coding for hybrid proteins.

Possibly, the sequence coding the hybrid protein mentioned in (a) is the coding sequence shown in fig. 5 or sequence presented as SEQ ID No.:1, and the sequence coding the hybrid protein mentioned in (b) is the sequence shown in fig. 2 or sequence presented as SEQ ID No.:3, and if the IRES sequence mentioned in (c) is the sequence presented in fig. 4 or sequence presented as SEQ ID No.:6. Preferably, the expression box according to the invention contains at least the nucleotides from 695 to 2538 of sequence presented as SEQ ID No.:5.

10 A further aspect of the invention is a two-cistron plasmid vector, characterised by the fact that it contains an expression box according to the invention. Here, possibly this plasmid vector is pVIF.

Another aspect of the invention is the application of the expression box according to the invention for the production of an expression vector for the gene therapy of circulatory deficiency in humans. In particular, the topic of the invention is the application of the pVIF plasmid in the production of a treatment for circulatory deficiency in humans.

The next aspect of the invention is a pharmaceutical composition for the treatment of circulatory deficiency in humans which contains an active ingredient and a pharmaceutically acceptable diluent, characterised by the fact that as the active ingredient it contains a two-cistron plasmid vector containing an expression box according to the invention. Here, the active ingredient is the pVIF plasmid.

Another aspect of the invention is also a method for the treatment or prevention of ischemic disorders or diseases of the human being comprising administering to a human in need thereof a medicament selected among expression box or pharmaceutical composition or vector according to invention, as defined above. Such medication can be administered either in or nearby to site of the ischemic tissue. Here, ischemic tissue is an ischemic myocardium.

Another aspect of the invention is also a method for revascularizing ischemic tissue or improving the vascular flow of ischemic tissue comprising administering a medicament selected from among an expression box or pharmaceutical composition or vector according

to invention, as defined above, either in or near to the site of the ischemic tissue. Here, ischemic tissue is an ischemic myocardium.

Medications in accordance with the invention may be administered intramuscularly or intravenously. For example, in the case of cardiac muscle ischemia intramuscular administration is possible, as is administration into the correct area of the cardiac capillaries.

THE VIRTUES OF THE INVENTION

The present invention unexpectedly facilitates a significant increase in the effectiveness of angiogenic gene therapy. Unexpectedly, using cross-species secretory sequences, an increase in the level of secretion of angiogenic factors was achieved. Unexpectedly, the presented vector induces the production of more mature and stabilised blood vessels *in vivo*, in comparison to a vector coding a single factor, i.e. only VEGF, and when applied in gene therapy (trials on patients) it causes more effective and faster improvements in the clinical state of the patient. An injection of a two-cistron vector entails a lesser introduction of foreign DNA as well as endotoxins, which may be contaminating the DNA preparation, than in the case of the administration of two single-gene vectors. By the same token one nullifies the danger of toxicity induced by the medication or other serious complications (fevers, shock and unconsciousness). Not meaningless are also the lower material costs needed for the production of one two-cistron vector. It is evident that the cost of isolation, purification, and endotoxin level testing is smaller than that of two single-gene vectors. Thanks to this the process of production of means may be more efficient with the same expenditure. This increases the availability of the medication.

The following examples will illustrate the invention further. These examples are, however, in no way intended to limit the scope of the present invention as obvious modifications will be apparent, and still other modifications and substitutions will be apparent to anyone skilled in the art.

The recombinant DNA methods employed in practising the present invention are standard procedures, well known to those skilled in the art, and described in detail, for example, in "Molecular Cloning" (Sambrook et al. 1989) and in "A Practical Guide to Molecular Cloning" (Perbal, 1984).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic of the expression box of the pVIF vector. The following acronyms were used: CMV- Cytomegaly virus promoter, ATG - start codon, TGA - stop codon, Igκ - mouse immunoglobulin signal sequence, VEGF – gene coding for the vascular endothelial growth factor, bFGF - gene coding the basic fibroblast growth factor, IRES - internal ribosome entry site.

Figure 2 shows the sequence of the ATG-Igκ-bFGF cloned into a pSEC/VEGF/IRES vector (NotI-ATG-Igκ-bFGF-XhoI). The primers used are underlined, the restriction sites used are shaded.

Figure 3 shows the bFGF sequence cloned into a pSEC (EcoRI-bFGF-XhoI) vector. The primers used are underlined.

Figure 4 shows the IRES sequence cloned into a pSEC/VEGF (EcoRI-IRES-NotI) vector. RBS-1 and RBS-2 primers are underlined, restriction sites used (EcoRI, Not I) are shaded.

Figure 5 shows the sequence ATG-Igκ-VEGF cloned into a pSEC/VEGF vector. Start and stop codons are in bold, fragments coding restriction sites are shaded.

For a better understanding of the essence of the invention, it is illustrated below through examples.

Example 1. The pVIF plasmid.

The pVIF construct is a plasmid expression vector coding angiogen-type factors. The cloned-in box contains two cistrons. It contains a gene coding VEGF₁₆₅ (PubMed X62568), the IRES sequence (based on a template cloned onto a pIRES-EGFP vector, Clontech, cat. #6064-1) as well as a sequence coding bFGF (Pubmed E02544). The IRES sequence stems from the encephalomyocarditis virus (ECMV) and ensures the translation of both cloned genes from one two-cistron mRNA. Both the VEGF and the FGF sequences are preceded by signal sequences in the box, which facilitate the secretion of the synthesized VEGF and FGF proteins. Figure 1 shows a schematic of the expression box in the pVIF vector.

Example 2. Obtaining the pVIF vector.

Cloning was carried out using sticky ends to the ATG triplet in accordance with the reading frame. Amersham reagents were used, and the vector was amplified in *E.coli*

bacteria, of the DH5 α strain. The isolation was carried out using the EndoFree Plasmid Mega Kit from Qiagen.

To obtain the pVIF vector, two supplementary vectors were used, pSEC/VEGF as well as an empty pSEC vector. The pSEC/VEGF vector was cloned earlier according to the description contained in Polish patent application P.339326, and it is a plasmid expression vector coding the VEGF165 isoform. It contains a signal sequence facilitating the secretion of the VEGF protein outside of the cell. The procedure was composed of three steps: (a) subcloning of the IRES sequence from the pMIG/IRES vector to a pSEC/VEGF vector, (b) cloning of the bFGF sequence onto a pSEC vector, (c) subcloning of the ATG - Ig κ - bFGF box from the pSEC/bFGF vector to the pSEC/VEGF/IRES vector.

A. Subcloning of the IRES sequence from the pMIG/IRES vector to a pSEC/VEGF vector. The IRES sequence was amplified using PCR from a template sequence cloned into a pMIG vector. The PCR primers were designed to contain the restriction sites for EcoRI and NotI:

15 RBS-1: 5' - AAGAATTCCAATTCCGCCCTCTCCCT - 3'

RBS-2: 5' - AAGCGGCCGCTTGTGGCAAGCTTATCATC - 3'

The PCR product was purified using the Clean-Up kit (DNA-Gdańsk), digested with EcoRI and NotI for 1 h at a temp. of 37 °C and isolated from an agarose gel using a QIAquick Gel Extraction Kit from Qiagen. The pSEC/VEGF vector was also digested with EcoRI and NotI, separated electrophoretically and isolated from the gel. Ligation was carried out over 16 h at 16 °C using T4 ligase (Amersham). Ligants were transformed into *E.coli* bacteria, and a clone selection was made on agar dishes with ampicillin. Grown clones were transferred to a liquid LB medium with ampicillin. They were incubated overnight at 37°C. Plasmids were isolated from the clones, which were then restriction-analysed. Thus the pSEC/VEGF/IRES vector was obtained.

B. Cloning of the bFGF sequence onto the pSEC vector

The sequence coding bFGF was amplified using a template of cDNA from cells of the HUVEC line (human urolimbical vein endothelial cell). The PCR primers were designed to contain the restriction sites for EcoRI and XhoI:

30 HbF-1: 5' - ATGAATTCGCCAGCATTGCCCGAGGAT - 3'

HbF-2: 5' – TTCTCGAGATTCAGCTCTTAGCAGACAT - 3'

The PCR product was purified using the Clean-Up kit (DNA-Gdańsk), digested with EcoRI and XhoI for 1 h at a temp. of 37 °C and isolated from an agarose gel using a
5 QIAquick Gel Extraction Kit from Qiagen. The pSEC vector was also digested with EcoRI and XhoI, separated electrophoretically and isolated from the gel. Ligation was carried out over 16 h at 16 °C using T4 ligase (Amersham). Ligants were transformed into *E.coli* bacteria, and a clone selection was made on agar dishes with ampicillin. Grown clones were transferred to a liquid LB medium with ampicillin. They were incubated overnight at
10 37°C. Plasmids were isolated from the clones, which were then restriction-analysed. Thus the pSEC/FGF vector was obtained.

C. Subcloning of the ATG - Igκ - bFGF box from the pSEC/bFGF vector onto the pSEC/VEGF/IRES vector

The ATG - Igκ- FGF box was cloned from the pSEC/FGF vector onto the
15 pSEC/VEGF/IRES vector. This fragment was amplified using PCR. A new primer was designed, which introduced a NotI restriction site, and the HbF-2 primer was also used. The sequence of the sense primer was as follows:

Not_IgK: 5' - AAGCGGCCGCACCATGGAGACAGACACA - 3'

From then, the procedure was carried out as before:: the PCR product was purified using
20 the Clean-Up kit (DNA-Gdańsk), digested with NotI and XhoI over 1 h at a temp. of 37 °C and isolated from an agarose gel using the QIAquick Gel Extraction Kit from Qiagen. The pSEC/VEGF/IRES vector was also digested with NotI and XhoI, separated electrophoretically and isolated from the gel. Ligation was carried out over 16 h at 16 °C using T4 ligase (Amersham). Ligants were transformed into *E.coli* bacteria, and a clone
25 selection was made on agar dishes with ampicillin. Grown clones were transferred to a liquid LB medium with ampicillin. They were incubated overnight at 37°C. Plasmids were isolated from the clones, which were then restriction-analysed. Thus the pSEC/VEGF/IRES/FGF (pVIF) vector was obtained.

The construct obtained underwent a detailed restriction analysis. Various combinations of
30 restrictases were used, as were several PCRs using various primers. The construct was also

sequenced. All analyses confirmed the correct cloning. A vector with a two-cistron VEGF/IRES/FGF expression box was obtained.

Example 3. *In vitro* assays

The constructed pVIF vector was inserted into tumour cells (LI line - murine sarcoma) and non-tumour cells (HCV-29 line - human urinary bladder epithelium) each with a low expression of VEGF and FGF growth factors. Using Western blotting, using specific monoclonal anti-VEGF and anti-FGF antibodies, we analysed the presence of these proteins in the medium surrounding the transfected cells. The analysis confirmed the expression of genes borne by the pVIF vector in the transfected cells, and that the synthesised VEGF and FGF proteins are secreted to the outside of the cell.

Example 4. *In vivo* studies

The studies were carried out in BALB/c strain mice (males). A skin angiogenesis test was performed. The mice received the vector subcutaneously at a dose of 10 µg per site. After 3 and 13 days following the injection new blood vessels were counted. It was concluded that the pVIF vector clearly induces the formation of blood vessels. The results were compiled in table 1.

Tabela 1. Comparison of effects

<i>In vivo</i> angiogenesis (mice) – the number of new blood vessels following an subcutaneous introduction of the vectors			
	pVIF	pVEGF	pFGF
3 days	21,33 +/- 3,77 n = 15	14,50 +/- 2,26 n = 14	12,13 +/- 1,96 n = 15
13 days	26,10 +/- 8,60 n = 17	18,20 +/- 4,5 n = 15	-

Example 5. Histochemical analysis

The mice used in the skin angiogenesis tests were used. Samples of subcutaneous tissue with the new blood vessels were taken. The purpose was to evaluate and compare the histological character of the blood vessels formed following the subcutaneous injection of the pVIF and pSEC/VEGF vectors. The results obtained prove that the pVIF vector

induces the formation of more mature and larger blood vessels than the pSEC/VEGF vector.

Example 6. Application of the pVIF vector in order to achieve angiogenesis in the cardiac muscle tissue of a patient with coronary disease.

5 The plasmid was used in a 52-year-old patient. The patient suffered from an unstable form of coronary disease of the III/IV class according to the CCS, without a heart attack, with arterial hypertension, hypercholesterolemia and atherosclerosis in his lower limbs.

During the pre-operation stress test he achieved a 5,5 METS result. The test was interrupted due to intense changes in the ST region in the effluent vessels from the front-
10 side and lower walls. The test result was described as singularly positive.

In the echocardiographic analysis, the ejection fraction was 65% (%EF=65). Coronography indicated multi-vessel coronary disease with a blocked circumflex artery (C_x), a blocked right coronary artery (RCA), thus it was only possible to bridge the left anterior descending artery (LAD).

15 During the operation, the left interior myocardial artery was grafted to the left anterior descending artery (LIMA to LAD) on a beating heart, without diverting blood externally. After performing the bridging to the coronary vessel, the bottom and side walls of the heart were injected with the pVIF plasmid solution. The cardiac muscle tissue was transfected directly, using an injection with a 0,33 mm needle.

20 No complications resulting from the gene transfection were noted, neither in the time during the operation, nor during the post-operational period. the patient was in a stable state throughout the time of the operation.

Presently, 3 months have passed since the operation, and the patient exhibits no atheros-
cardiac illnesses, and has functional breathing and circulation. In a SPECT analysis 2
25 weeks following the operation, perfusion was noted which was related to the insertion of the bridge to the LAD, and afterwards perfusion was noted in segments transfected with the pVIF plasmid in an examination performed 1 month following the operation. The patient's state is improving continually. In further examinations, revascularizing ischemic myocardial tissue was observed, which was exhibited by improvement of the vascular flow
30 of the ischemic myocardium.

In summary, one may concluded that the application of the pVIF plasmid did not increase the risk of the operation. The patient bore the operation very well. No post-operational complications were observed. The patient does not report more coronary problems. His life comfort level has increased, along with his stress tolerance, and his
5 requirement for nitrates has lessened.

CLAIMS

1. Expression box composed of a CMV promoter as well as an SV40 polyadenylation signal operationally connected to a gene complex containing:
 - a) a sequence coding a hybrid protein containing a secretory sequence stemming from immunoglobulin bound to the VEGF₁₆₅ sequence with a removed native secretory sequence,
 - b) a sequence coding the hybrid protein containing a secretory sequence stemming from immunoglobulin bound to the bFGF sequence,
 - c) an IRES sequence located between the mentioned sequences coding hybrid proteins.
2. Expression box according to claim 1, characterised in that the sequence coding the hybrid protein mentioned in (a) is the coding sequence presented in Figure 5 or sequence presented as SEQ ID No.:1.
3. Expression box according to claim 1, characterised in that the sequence coding the hybrid protein mentioned in (b) is the coding sequence presented in Figure 2 or sequence presented as SEQ ID No.:3.
4. Expression box according to claim 1, characterised in that the IRES sequence mentioned in (c) is the sequence presented in Figure 4 or sequence presented as SEQ ID No.:6.
5. Expression box according to claim 1, characterised in that it contains at least the nucleotides from 695 to 2538 of sequence presented as SEQ ID No.:5.
6. Two-cistron plasmid vector, characterised in that it contains an expression box composed of a CMV promoter as well as an SV40 polyadenylation signal connected operationally to a gene complex containing:
 - a) a sequence coding a hybrid protein containing a secretory sequence stemming from immunoglobulin bound to the VEGF₁₆₅ sequence with a removed native secretory sequence,
 - b) a sequence coding the hybrid protein containing a secretory sequence stemming from immunoglobulin bound to the bFGF sequence,
 - c) an IRES sequence located between the mentioned sequences coding hybrid proteins.

7. Plasmid vector according to claim 6, characterised in that the sequence coding the hybrid protein mentioned in (a) is the coding sequence presented in Figure 5 or sequence presented as SEQ ID No.:1.
8. Plasmid vector according to claim 6, characterised in that the sequence coding the hybrid protein mentioned in (b) is the coding sequence presented in Figure 2 or sequence presented as SEQ ID No.:3.
9. Plasmid vector according to claim 6, characterised in that the IRES sequence mentioned in (c) is the sequence presented in Figure 4 or sequence presented as SEQ ID No.:6.
10. Plasmid vector according to claim 6, characterised in that the sequence coding the hybrid proteins contains at least the nucleotides from 695 to 2538 of sequence presented as SEQ ID No.:5.
11. Plasmid vector according to claim 6, characterised in that it is a pVIF plasmid.
12. The use of the expression box composed of the a CMV promoter as well as an SV40 promoter connected operationally to a gene complex containing:
 - a) a sequence coding a hybrid protein containing a secretory sequence stemming from immunoglobulin bound to the VEGF₁₆₅ sequence with a removed native secretory sequence,
 - b) a sequence coding the hybrid protein containing a secretory sequence stemming from immunoglobulin bound to the bFGF sequence,
 - c) an IRES sequence located between the mentioned sequences coding hybrid proteins,for the production of an expression vector for use in gene therapy of ischemic states in humans.
13. Use according to claim 12, characterised in that the sequence coding the hybrid protein mentioned in (a) is the coding sequence presented in Figure 5 or sequence presented as SEQ ID No.:1.
14. Use according to claim 12, characterised in that the sequence coding the hybrid protein mentioned in (b) is the coding sequence presented in Figure 2 or sequence presented as SEQ ID No.:3.

15. Use according to claim 12, characterised in that the IRES sequence mentioned in (c) is the sequence presented in Figure 4 or sequence presented as SEQ ID No.:6.
16. Use according to claim 12, characterised in that the sequence coding the hybrid proteins contains at least the nucleotides from 695 to 2538 of sequence presented as SEQ ID No.:5.
17. Use of the pVIF plasmid to produce medication for the treatment of ischemic states in humans.
18. A pharmaceutical composition for the treatment or prevention of ischemic disorders or diseases of the human being comprising an active ingredient and a pharmaceutically acceptable carrier or diluent characterised in that as the active ingredient it contains a two-cistron plasmid vector containing a CMV promoter as well as an SV40 promoter connected operationally to a gene complex containing:
 - a) a sequence coding a hybrid protein containing a secretory sequence stemming from immunoglobulin bound to the VEGF₁₆₅ sequence with a removed native secretory sequence,
 - b) a sequence coding the hybrid protein containing a secretory sequence stemming from immunoglobulin bound to the bFGF sequence,
 - c) an IRES sequence located between the mentioned sequences coding hybrid proteins.
19. A pharmaceutical composition according to claim 18, characterised in that the sequence coding the hybrid protein mentioned in (a) is the coding sequence presented in Figure 5 or sequence presented as SEQ ID No.:1.
20. A pharmaceutical composition according to claim 18, characterised in that the sequence coding the hybrid protein mentioned in (b) is the coding sequence presented in Figure 2 or sequence presented as SEQ ID No.:3.
21. A pharmaceutical composition according to claim 18, characterised in that the IRES sequence mentioned in (c) is the sequence presented in Figure 4 or sequence presented as SEQ ID No.:6.
22. A pharmaceutical composition according to claim 18, characterised in that the sequence coding the hybrid proteins contains at least the nucleotides from 695 to 2538 of sequence presented as SEQ ID No.:5.

23. A pharmaceutical composition according to claim 18, characterised in that the active ingredient is the pVIF plasmid.
24. A method for the treatment or prevention of ischemic disorders or diseases of the human being comprising administering to a human in need thereof an expression box according to claim 1 to 5.
25. A method of claim 24 wherein an expression box is administered either in or near to site of the ischemic tissue.
26. A method of claim 24 wherein ischemic tissue is a ischemic myocardium.
27. A method for the treatment or prevention of ischemic disorders or diseases of the human being comprising administering to a human in need thereof a vector according to claim 6 to 11.
28. A method of claim 27 wherein a vector is administered either in or nearby to site of the ischemic tissue.
29. A method of claim 27 wherein ischemic tissue is a ischemic myocardium.
30. A method for the treatment or prevention of ischemic disorders or diseases of the human being comprising administering to a human in need thereof a pharmaceutical composition according to claim 18 to 23.
31. A method of claim 30 wherein a pharmaceutical composition is administered either in or nearby to site of the ischemic tissue.
32. A method of claim 30 wherein ischemic tissue is a ischemic myocardium.
33. A method for revascularizing ischemic tissue or improving the vascular flow of ischemic tissue comprising administering a expression box according to claim 1 to 5 or a vector according to claim 6 to 11 or a pharmaceutical composition according to claim 18 to 23 either in or nearby to site of the ischemic tissue.
34. A method of claim 33 wherein ischemic tissue is a ischemic myocardium.

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5' -

3'

CMV	ATG	Igκ	VEGF	TGA	IRES	ATG	Igκ	bFGF	TGA
-----	-----	-----	------	-----	------	-----	-----	------	-----

Figure 1

5' -

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TGTTTCTTTTTTGAACGATTGGAATCTAATAACTACAATACTTACCGGTCAAGGAAATACA
CCAGTTGGTATGTGGCACTGAAACGAACTGGGCAGTATAAACTTGGATCCAAAACAGGACC
TGGGCAGAAAGCTATACTTTTTCTTCCAATGTCTGCTAAGAGCTGAATCTCGAGAA - 3'

Figure 2

5' -

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CGAACTGGGCAGTATAAACTTGGATCCAAAACAGGACCTGGGCAGAAAGCTATACTTTTTTC
TTCCAATGTCTGCTAAGAGCTGAATCTCGAGAA - 3'

Figure 3

5' -

AAGAATTCCAATTCGGCCCTCTCCCTCCCCCCCCCTAACGTTACTGGCCGAAGCCGCTT
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- 3'

Figure 4

5'

ATGGAGACAGACACACTCCTGCTATGGGTA**CTGCTGCTCTGGGTTC**CAGGTTCC
CACTGGTGACGCGGCC**CCAGCCGGCCAGGCGCGCGCCGTACGAAGCTTGGTA**
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Figure 5

SEQUENCE LISTING

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