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Il Farmaco 55 (2000) 165–167

IL FARMACO

Recombinant production of PIGF-1 and its activity in animal models

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Abstract

In this paper we review current knowledge on placenta growth factor (PIGF) and summarise our data on its recombinant production in bacteria and its activity. PIGF and vascular endothelial growth factor (VEGF) are both angiogenic factors belonging to the platelet-derived growth factor (PDGF) family. PIGF is a dimeric glycoprotein which shares a number of biochemical and functional features with VEGF. The aminoacidic similarity between the two factors is high (about 50%) in the PDGF-like domain. By alternative splicing of the PIGF mRNA, three forms of PIGF protein are generated which are named PIGF-1, PIGF-2 and PIGF-3. We have focused our attention on form 1 of human PIGF (PIGF-1). A large quantity of active recombinant PIGF-1 has been obtained using a bacterial expression system. By optimising the fermentation and purification it was possible to produce about 140 mg/l of culture of active PIGF-1, which is potentially suitable for a pharmaceutical use. The angiogenic activity of two different batches of bacteria-derived PIGF-1 obtained in our laboratory was demonstrated in chick chorionallantoic membrane assays. Finally, in preliminary studies we have shown that bacteria-derived PIGF-1 has a protective effect against myocardial lesions induced by isoprenaline in rat and rabbit.

Keywords: Placenta growth factor; Angiogenesis; Myocardiac infarct

1. Introduction

Angiogenesis is essential for development, reproduction, tissue regeneration and remodelling [1]. Therapeutic angiogenesis may be useful in those pathologies where its stimulation may have a beneficial effect, such as myocardial/peripheral ischemia, myocardial infarction and wound healing.

VEGF is an angiogenic factor specific for the endothelial cells [2–4]. PIGF is a secreted placenta-derived homodimeric glycoprotein that shares substantial structural similarity with VEGF [5,6]. Due to this strong similarity PIGF and VEGF can form heterodimers [7–9]. Furthermore, PIGF binds to one of the two VEGF receptors, namely Flt-1, that is essential for the organisation of embryonic vasculature, as demonstrated by the knock out of the corresponding mouse gene [10,11]. Moreover, Flt-1 mediates the migration of monocytes and also the production of tissue factor [12,13], which seems to have a role in angiogenesis [14,15].

Angiogenesis induced by recombinant, cell-derived, human PIGF has been demonstrated both in chick chorionallantoic membrane (CAM) and in avascular rabbit cornea assays [16].

By alternative splicing of the PIGF mRNA, three forms of PIGF protein are generated which are named PIGF-1 [5], PIGF-2 [6,17] and PIGF-3 [18]. Only the PIGF-2 protein has the property of binding to heparin [17].

We have focused our attention on PIGF-1 protein and have succeeded in producing a large quantity of active recombinant human PIGF-1 using a bacterial expression system. In animal models this recombinant protein is able to protect the heart against myocardial lesions induced by isoprenaline.

2. Results

2.1. Production and purification of the PIGF-1 protein

By polymerase chain reaction (PCR), the region of the human PIGF-1 gene coding for the mature protein

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0014-827X/00/$ - see front matter
PII: S0014-827X(00)00012-4

was cloned into a prokaryotic expression vector as described [5]. Then, the recombinant vector was used to transform a DE3 coli strain and the synthesis of PIGF-1 was induced by 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). Inclusion bodies were purified from the induced bacteria and refolded for 20 h at 20°C.

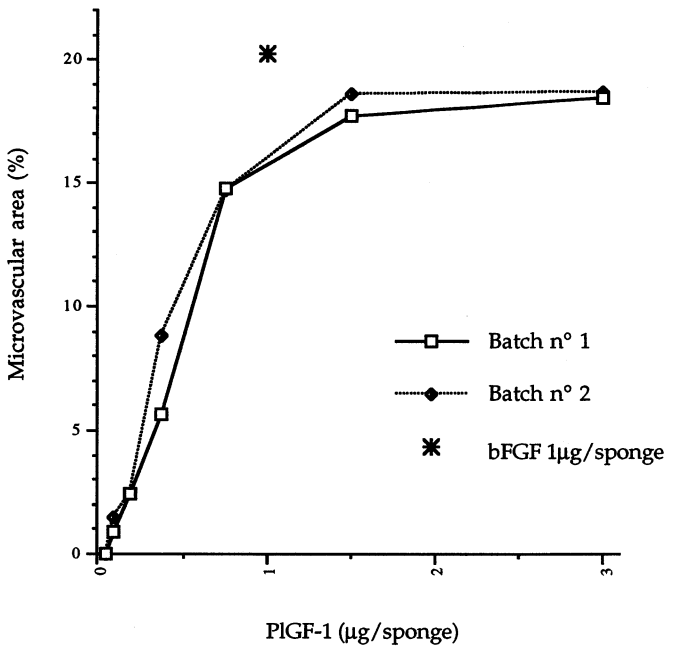

Fig. 1. Angiogenic activity of recombinant PIGF-1. Various quantities of two batches of bacteria-derived PIGF-1 were adsorbed onto 1 mm^3 gelatin-sponges, which were implanted on top of the CAM. After 12 days, the regions of CAMs surrounding sponges were cut off and coloured and angiogenesis was quantified by the 'point counting' method (20). Basic fibroblast growth factor (bFGF, 1 μg) was used as positive control. Five eggs were used for each point.

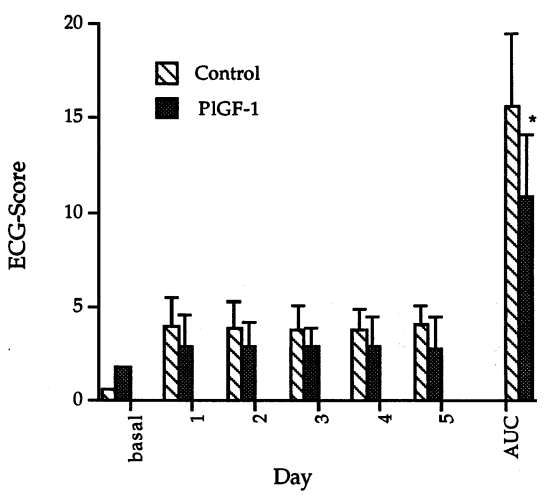

Fig. 2. Effect of PIGF-1 on evolution of the ECG-changes induced by isoprenaline. The ECG-changes were scored according to the following seven-point scale: 0, no lesions; 1, S-wave prominence; 2, T-wave prominence; 3, depression of the descending branch of T-wave; 4, S-wave enlargement; 5, T-wave inversion; 6, Q-wave prominence. AUC represent the area under curve formed by the ECG-score from day 1 to day 5. Symbol '*' indicates that the mean value of the group (10 animals) is statistically different with respect to control at $P < 0.05$.

The refolded PIGF-1 protein was purified by anion-exchange chromatography followed by reverse-phase chromatography. Final recovery of active PIGF-1 purified in our laboratory was about 140 mg/l. The identity of the protein was proven by various assays, such as immunoblotting, SDS-PAGE gel electrophoresis under reducing and unreducing conditions, bidimensional electrophoresis, reverse-phase HPLC and amino-terminal sequencing. Purity of the PIGF-1 protein respect to the host (bacterial) proteins was also determined by an immunological test. The quantity of the residual host proteins was less than 1.6 ng/mg of PIGF-1 in the eight batches analysed.

2.2. Activity of the PIGF-1 protein

Angiogenic activity of two batches of purified PIGF-1 was tried in a CAM assay [19] and the angiogenesis was quantified by a morphometric methods named 'point counting'[20]. Briefly, sections of CAM were analysed microscopically by a grid with 144 intersection points. Data were expressed as a percentage of intersection points occupied by vessels in cross-section (percentage of microvascular area). Fig. 1 shows the rate of angiogenesis induced by various quantities of PIGF-1. Two batches of PIGF-1 induced an angiogenic response very similar in potency (EC_{50} between 0.3 and 0.5 μg/sponge).

Angiogenesis may have a beneficial effect in myocardial ischemia or infarction. Thus, PIGF-1 could have suchlike pharmacological effect. This hypothesis was tested in animal models of cardiac ischemia based on the administration of isoprenaline. This amine produces, in animals, ischemic lesions similar to those observed in myocardial infarction in humans [21–23]. These experiments were conducted on rabbits, a single dose of PIGF-1 (160 μg/kg) or vehicle alone was given by intravenous route for 4 consecutive days; namely from day 1 to day 4. Isoprenaline was given by a subcutaneous route on day 1 and day 2. PIGF-1 caused a significant reduction ($P < 0.01$), with respect to the control animals, of the serum level of lactate dehydrogenase on day 2 and day 4, while on day 4, a significant decrease was also revealed for aspartate aminotransferase ($P < 0.05$) and creatine kinase ($P < 0.01$) enzymes. The electrocardiographic (ECG) changes indicative of major ischemic damage, such as T-wave inversion, S-wave enlargement and Q-wave prominence, were more evident in vehicle-treated animals than in PIGF-treated animals. When the ECG changes were scored according to the seven-point scale reported in the legend of Fig. 2 and the area under curve (AUC) was calculated, a significant ($P < 0.05$) reduction of the

Table 1
Macroscopic evaluation of ischemic lesions in the myocardium [a]

Animal group	Number of animals	Score
Vehicle-treated	10	Mean = 3.2 SD = ±1.55
PIGF-1 treated	10	Mean = 1.1* SD = ±0.56

[a] The lesions were scored according to the following seven-point scale: 0, no lesions; 1, small lesions (<2 mm) in the left ventricle and in the apex; 2, less than three large lesions (>2 mm) in the left ventricle and in the apex; 3, small lesions in the atria; 4, less than three large lesions in the atria; 5, three or more large lesions in the ventricles and apex; 6, three or more large lesions in the atria. Symbol '*' indicates that the mean value of the group is statistically different with respect to control at $P<0.05$. SD, standard deviation.

Table 2
Incidence of some heart histological alterations [a]

Tissues and diagnoses	Vehicle-treated group (%)	PIGF-1-treated group (%)
Left ventricle		
Vacuolar degeneration	80	30
Myocardial necrosis	30	30
Right ventricle		
Vacuolar degeneration	50	20
Myocardial necrosis	50	20
Septum		
Vacuolar degeneration	20	0
Myocardial necrosis	40	0
Apex		
Vacuolar degeneration	60	20
Myocardial necrosis	40	10

[a] Ten hearts for each group were examined.

AUC was noted. The observation that the electrocardiographic pattern of PIGF-treated animals was less severe than in control animals was confirmed by a macroscopic evaluation of the necrotic areas of the hearts at day 5. Table 1 shows that the score expressing the severity of cardiac necrosis is significantly lower in PIGF-treated animals. Moreover, the beneficial effect of PIGF-1 on the isoprenaline-induced ischemia was also confirmed by the histophatological analysis of hearts. In fact, as shown in Table 2, administration of PIGF-1 significantly reduces the incidence of vacuolar degeneration and myocardial necrosis with respect to the vehicle-treated animals.

The same kind of study was carried out on rats using three doses of PIGF-1: namely 20, 40 and 160 μg/kg. Also, in this species, PIGF-1 is able to counteract the progression of the myocardial lesions at the two higher doses (data not shown).

3. Discussion

Data shown above indicate that PIGF-1 may prevent tissue necrosis in the ischemic myocardium and reduce infarct size. Concerning the mechanism by which PIGF explicates this protective effect against cardiac ischemia, present data do not allow an hypothesis to be formulated, since the formation of new arterioles surrounding the necrotic area was not studied. However, a pharmacodynamic effect on the heart can be excluded, because PIGF-1 does not influence the tachycardia induced by isoprenaline both in rabbit and rat (data not shown).

Thus, if these preliminary data are confirmed, PIGF-1 could be considered as a potential drug in therapy for heart ischemia and/or infarct.

Such a use has also been proposed for VEGF. However, it may be that PIGF could have less side-effects than VEGF. In fact PIGF, with respect to VEGF, has no permeability activity [7]. In addition, it is expressed only in few tumours and in such, namely thyroid and germ cell tumours, it is not correlated with tumour progression [24] or tumour neoangiogenesis [25], respectively.

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