Comparison of protein with DNA therapy for chronic myocardial ischemia using fibroblast growth factor-2

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Abstract

Objective: Treatment of coronary disease by growth factors has become an increasingly used strategy for otherwise untreatable patients and is subject to a number of clinical studies. The aim is to stimulate the development of a sufficient collateral circulation and hereby to rescue cardiac function. The objective of our study was to compare the effectiveness of fibroblast growth factor-2 (FGF-2) as protein and as naked plasmid DNA in a porcine model of chronic myocardial ischemia.

Materials and methods: A severe stenosis of the left anterior descending artery (LAD) artery was created in healthy pigs. After 1 week, perfusion and regional and global contractility was assessed at baseline at rest and under stress. Afterwards, recombinant FGF-2 (n = 6) or naked plasmid DNA encoding FGF-2 (n = 7) was intramyocardially injected into the LAD territory. Control animals were left untreated (n = 5). After 3 months, the animals were re-examined and underwent immunohistologic analysis. One animal received an Enhanced Green Fluorescent Protein plasmid. Results: Plasmid-dependent protein synthesis was present in cardiomyocytes. FGF-2 protein as well as plasmid injections resulted in an increased number of capillaries and of arterioles compared with untreated ischemia. The improvement of the regional myocardial blood flow by FGF-2 plasmid therapy at rest might however indicate the effectiveness of the DNA application for the induction of a collateral circulation. A benefit from FGF-2 plasmid therapy was revealed with regard to regional contractility. Systemic hemodynamics were partially improved following plasFGF-2 treatment.

Conclusions: In this porcine model of chronic myocardial ischemia, intramyocardial injection of FGF-2 plasmid was more effective than of FGF-2 protein in improving regional perfusion and contractility compared to untreated ischemia. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Coronary disease; Fibroblast growth factor-2; Gene expression; Angiogenesis; Perfusion; Contractility

1. Introduction

Treatment of coronary disease using growth factors has become an increasingly used strategy for otherwise untreatable patients and is the subject of many clinical studies. The goal is to stimulate the development of sufficient collateral circulation and hereby to resume normal regional and global cardiac function. Fibroblast growth factor-2 (FGF-2, basic fibroblast growth factor) is one of the most widely used factors. It is involved in the control of growth and differentiation of epithelial, mesenchymal, and neuroectodermal cells [1,2]. In addition, it promotes intercellular contact and communication [3]. During early postnatal coronary development, FGF-2 regulates the growth of capillaries as well as arterioles and is responsible for the normal development of the vessel tree [4].

Intramyocardial injection of agents strictly targets the area in need. Protein application is characterized by a defined dose but limited tissue half-life, whereas gene injection results in poorly predictable amount of protein production but prolonged presence of the growth factor. Results of clinical trials with intramyocardial delivery of a VEGF\textsubscript{165} DNA plasmid [5] or, as in our previous study, of free recombinant FGF-1 [6,7] suggest, that both applications can be successfully employed for treatment of chronic myocardial ischemia.

The objective of our present study was to compare the effectiveness of intramyocardial injection of protein compared to naked plasmid DNA for FGF-2 with regard to vessel growth (angiogenesis and arteriogenesis), perfusion, and cardiac function in a porcine model of chronically myocardial ischemia.

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2. Material and methods

2.1. Preparation of plasmid DNA

The coding sequences of human FGF-2 or Enhanced Green Fluorescent Protein (EGFP) were cloned into a mammalian expression vector (pCI-neo; Promega, Madison, WI) under control of the cytomegalovirus promoter. The plasmids were amplified in Escherichia coli (XL-1 Blue; Stratagene, La Jolla, CA), and retrieved and purified using the QIAGEN Plasmid Giga Kit (QIAGEN, Valencia, CA) according to the manufacturers’ instructions.

2.2. Animals and anaesthesia

All animal procedures were performed in compliance with the ‘Principles of Laboratory Animal Care’, the ‘Guide for the Care and Use of Laboratory Animals’, National Institute of Health publication 85-23, revised 1985, and the German ‘Law for Animal Protection’. Pigs of ‘German Landrace’ weighing 24–30 kg were premedicated, anaesthetized, and monitored as previously described [8]. Sufficient analgesics as well as acetyl salicylic acid and antibiotics were given after the first and second operation.

To mimic clinical coronary artery disease, we employed a model of chronic myocardial ischemia [9]. In the first operation, an operative stenosis of the left anterior descending artery (LAD) was created in the ischemic experimental groups. One week later (second operation), the animals were studied by analyzing different parameters (see Section 2.2.3). Afterwards, pigs were designated to one of three different experimental groups. After 12 weeks (third operation), the animals were re-examined (same parameters as before) and sacrificed. One additional pig received the EGFP plasmid and was sacrificed 2 days later.

2.2.1. First operation

The heart was exposed via an anterolateral minithoracotomy. The LAD was carefully dissected and isolated immediately distal to the bifurcation of the first diagonal branch (D1) over 1–2 cm to accept an ultrasonic transit time (UTT) flow probe (Transonic Systems, Inc, Ithaca, NY) recording downstream flow through the LAD. A severe LAD stenosis (immediately distal to D1) was created proximal to the flow probe by the arterial puncture needle technique [9] to produce an area at risk of about 25% of the LV anterior free wall.

The blood flow distal of the stenosis was reduced to about 50% as assessed by UTT and coronarography. The wound was closed in layers, and the animals were allowed to recover.

2.2.2. Second operation

Through a re-minithoracotomy, the pericardium was opened and the heart was reexposed 7 days after the onset of chronic ischemia under the same conditions as described above. The monitoring was performed, as reported elsewhere [10].

Once angiography and UTT flow probe data confirmed the presence of chronic ischemia (severe LAD stenosis with blood flow reduction, see above); baseline measurements of segmental myocardial shortening, left ventricular stroke work index, and time derivative of maximal left ventricular pressure were assessed as described below at rest and under stress. Stress was exerted by intravenous application of dobutamine in increasing doses up to 20 μg/min per kg. In addition, fluorescent microspheres were injected for examination of regional perfusion as described below. Animals were designated to one of three groups. To define this area at risk the LAD was occluded for 10 s prior to treatment. The pigs received therapy or were left untreated (see Section 2.2.3). The thorax was closed, and the pigs were allowed to recover.

2.2.3. Experimental groups

Pigs received 500 μg human recombinant Fibroblast growth factor-2 (Pepro Tech Inc., Rocky Hill, NJ) [recFGF-2, n = 6]) or 2 mg FGF-2 plasmid [plasFGF-2, n = 7]. Both agents were supplemented with 400 IE Heparin and diluted in 0.9% sodium chloride to a total volume of 2.8 ml. Out of this amount, 15 aliquots were injected equidistantly into the myocardium across the ischemic area. The third group did not receive injections and was left untreated [ischemia, n = 7].

2.2.4. Third operation

After 3 months, a sternotomy was performed. The animals were reassessed similarly to the second operation and sacrificed. The hearts were removed and cut in 5 mm transversal sections. Samples were taken from the ischemic area as well as from the posterior wall and fixed in paraformaldehyde.

2.3. Parameters

2.3.1. Enhanced green fluorescent protein

Myocardial samples were put into 0.1 M potassium phosphate buffer pH 7.6 containing 15% sucrose and preserved at −20 °C. The nuclei were counterstained using propidium iodide (0.5 μg/ml) in cryosections, and fluorescence was observed using a fluorescence microscope (Zeiss, Germany).

2.3.2. Vessel counting

Myocardial sections were fixed in 4% formaldehyde in phosphate buffer immediately after removal and embedded in paraffin. Sections were taken from the ischemic area or, for control purposes, from the corresponding myocardium of healthy untreated weight-matched pigs (n = 5). Slices were pretreated with methanol, H2O2, and pepsin (Sigma, Taufkirchen, Germany), and double-stained after blocking with the appropriate serum. Endothelial cells
were identified by successive incubation with anti-Von Willebrand Factor (DAKO, Hamburg, Germany), biotinylated swine anti-rabbit F(ab’2) fragment (DAKO), Streptavidin-biotinylated alkaline phosphatase-complex (DAKO), and Fast Blue (Sigma). For staining of smooth muscle cells, anti-Smooth muscle actin (DAKO), biotinylated rabbit anti-mouse F(ab’2) fragment (DAKO), and Streptavidin-biotinylated Horseradish Peroxidase-complex (DAKO) were used followed by development with DAB (Sigma). Counting of capillaries (400-fold enlargement) and arterioles (200-fold enlargement) was performed by trained observers blinded to the experimental conditions. Arterial structures with more than three layers of smooth muscle cells were considered arteries and were excluded. For each animal, 40 visual fields from different sections were counted.

### 2.3.3. Regional myocardial blood flow (RMBF)

Regional perfusion of the territories of the LAD and left circumflex artery was measured by fluorescent microspheres (Molecular Probes, Eugene, OR) based on the arterial reference sample technique [8,11]. The microsphere suspensions (15 ± 0.1 μm; density of 1.07 g/ml) were injected into the left atrium under stable hemodynamic conditions. The reference samples were withdrawn from the internal carotid artery over a 2 min period at a rate of 10 ml/min starting 5 s before the injection of microspheres. Microspheres were injected at rest and under stress in all animals at the second (before application of therapy) and third operation (Harvard apparatus, South Natick, MA). Different colours were used for the four study points in each animal.

At the end of study, myocardial samples were taken from the ischemic area. The microspheres were retrieved from the tissue according to the manufacturers’ instructions and the resulting fluorescence was measured.

### 2.3.4. Segmental myocardial shortening (SMS)

Assessment of segmental myocardial shortening (SMS) was performed at rest and under stress in all animals using ultrasonic crystals (Transonic Systems, Inc., Ithaca, NY), as previously described [8].

SMS was calculated as follows:

\[
\% \text{ SMS} = \frac{\text{EDL} - \text{ESL}}{\text{EDL}} \times 100
\]

where EDL and ESL are end-diastolic length and end-systolic length, respectively.

SMS were analyzed at three different times: before stenosis (first operation), after 1 week chronic ischemia (second operation, baseline), and after 3 months ischemia (third operation).

### 2.3.5. Hemodynamic Measurements

Hemodynamic measurements of LV pressure, maximum of the first derivative of left ventricular pressure at a defined left atrial pressure (dp/dt\text{max}) measured by Millar catheter (Millar, Houston, TX), and maximal left ventricular stroke work index (LVSWI\text{max}) were monitored continuously and recorded at rest and under stress in all experimental groups. Stress was induced by IV application of dobutamine for 10 min at a dose of 10 μg kg\text{−1} min\text{−1}, followed by a dose of 20 μg kg\text{−1} min\text{−1} IV for another 10 min. Left ventricular stroke work was normalized for heart frequency (HF) and body weight (BW) and calculated as:

\[
\text{LVSWI} = \frac{(\text{MAP} - \text{LAP}) \times \text{CO}}{\text{HR} \times \text{BW}} \times 0.133 \times 1000
\]

where LVSWI is the LV stroke work index (mJ/kg); MAP the mean arterial pressure (mmHg); LAP the left atrial pressure (mmHg); CO the cardiac output (l/min); HR the heart rate (beats/min); BW the body weight (kg).

The maximal-achieved LVSWI (LVSWI\text{max}) was used for comparison between the experimental groups.

All parameters were assessed after 1 week (second operation, baseline) and three months of chronic ischemia (third operation) at rest and under stress conditions.

### 2.3.6. Statistical analysis

Data were analyzed based upon Wilcoxon’s signed rank test to compare paired data, and Mann–Whitney U-test to compare unpaired data for non-normally distributed data, as appropriate (SPSS-vers. 10.01). Results are expressed as mean ± standard deviation (SD). A P-value less than 0.05 was considered statistically significant. Only data were used from animals who survived the entire 13-week observation period and suffered no apparent infarction.

### 3. Results

#### 3.1. Gene expression in vivo

One animal was injected with the EGFP plasmid and sacrificed after 2 days. A strong patchy presence of the protein was apparent in the treated area indicating an efficient transfection of cardiomyocytes (Fig. 1). Samples from non-ischemic untreated myocardium did not show green fluorescence (not shown).

#### 3.2. Angiogenesis and arteriogenesis

Myocardial sections from the ischemic area were double-stained for von Willebrand factor and Smooth muscle actin. Capillaries and arterioles were subsequently counted (Fig. 2). Chronic myocardial ischemia resulted in a significant decrease of the number of capillaries after three months of ischemia compared to values of myocardium from normal healthy pigs (P = 0.008). Injection of FGF-2 protein or plasmid increased the number of capillaries compared to ischemia (P = 0.009 and P = 0.003, respectively). The FGF-2 groups did not differ from each other (P = 1.0).
There was no evidence of angioma formation macroscopically as well as microscopically. The number of arterioles was not reduced in the ischemic compared to normal healthy myocardium ($P = 0.151$). In contrast, treatment with recFGF-2 or plasFGF-2 resulted in arteriogenesis (compared to the ischemic control, $P = 0.03$ for both groups). In addition, the number of arterioles after plasFGF-2 therapy was higher than in normal myocardium ($P = 0.03$). However, there was no difference between the treated groups ($P = 0.628$) (Fig. 3b).

### 3.3. Regional myocardial blood flow (RMBF)

Regional perfusion in the ischemic area was assessed using fluorescent microspheres employing the arterial reference sample technique after 1 week of ischemia before the application of growth factors (baseline), and three months later. The analysis of the ischemic LAD area indicated better regional blood flow at rest following the injection of plasFGF-2 ($P = 0.043$ versus baseline), whereas no treatment or recFGF-2 did not render improvement ($P = 0.735$ and $P = 0.068$ versus baseline, respectively). However, there were no significant changes observed under stress conditions (ischemia: $P = 0.917$, recFGF-2: $P = 0.6$, plasFGF-2: $P = 0.463$ versus baseline) (Table 1).

### 3.4. Regional contractility

Myocardial ischemia of the LAD territory resulted in a deterioration of SMS after 1 week in all animals (20.94 ± 5.58 pre-ischemic versus 12.68 ± 5.05 after 1 week at rest, $P = 0.001$). There were no differences (Fig. 3a). There was no evidence of angioma formation macroscopically as well as microscopically.

The number of arterioles was not reduced in the ischemic compared to normal healthy myocardium ($P = 0.151$). In contrast, treatment with recFGF-2 or plasFGF-2 resulted in arteriogenesis (compared to the ischemic control, $P = 0.03$ for both groups). In addition, the number of arterioles after plasFGF-2 therapy was higher than in normal myocardium ($P = 0.03$). However, there was no difference between the treated groups ($P = 0.628$) (Fig. 3b).
between the three experimental groups (ischemia: $P = 0.109$ versus recFGF-2, $P = 0.775$ versus plasFGF-2, recFGF-2: $P = 0.063$ versus plasFGF-2). Thus, the baseline

After 1 week was reduced to $60.97\% \pm 16.82$ of the pre-ischemic values in all ischemic animals (Fig. 4).

After 3 months, SMS in untreated pigs had further decreased at rest ($P = 0.027$ versus 1-week baseline). Regional contractility in recFGF-2-treated myocardium remained unchanged ($P = 0.6$ versus baseline), whereas plasFGF-2-therapy resulted in an improvement ($P = 0.028$ versus baseline) at rest (Fig. 5a).

Examination under stress conditions exerted by $20 \mu$g/min per kg dobutamine revealed an increase of SMS values in the plasFGF-2 group compared to the ischemic group ($P = 0.047$). Treatment with recFGF-2 produced no significant change ($P = 0.1$ versus ischemia). One animal from the ischemic group and two animals from the plasFGF-2 group did not tolerate the rising doses of dobutamine up to $20 \mu$g/min per kg, and therefore those experiments had to be cut short and the corresponding data were excluded (Fig. 5b).

3.5. Maximal left ventricular stroke work index (LVSWI_max)

After 3 months, there was a decline of the LVSWI_max (Table 2) at rest in the ischemic and recFGF-2 groups (ischemia, $P = 0.018$, recFGF-2, $P = 0.028$ versus baseline), which was not observed in the plasFGF-2 group ($P = 0.091$ versus baseline). PlasFGF-2 therapy achieved higher LVSWI_max values compared to recFGF-2 at rest ($P = 0.032$). However, the groups receiving therapy did not differ from the untreated ischemic control after 3 months (recFGF-2, $P = 0.668$, plasFGF-2, $P = 0.565$).

No treatment was able to improve LVSWI_max under stress conditions in comparison with the ischemic control (recFGF-2, $P = 0.361$, plasFGF-2, $P = 0.855$). (Table 2).

3.5.1. Maximum of the first derivative of left ventricular pressure ($dp/dt_{max}$)

Therapy with plasFGF-2 injection resulted in higher $dp/dt_{max}$ values at rest compared to the untreated control.
P = 0.012), whereas recFGF-2 therapy did not show this effect (P = 0.15 versus ischemia). The treatment groups did not differ from one another (P = 0.391).

There was no improvement of dp/dt\text{max} in comparison with the ischemic group under stress conditions (recFGF-2, P = 0.317, plasFGF-2, P = 0.182) (Table 2).

4. Discussion

Growth factor therapy for ischemic diseases is postulated to stimulate the establishment of a sufficient collateral circulation and hereby to maintain myocardial function. In this study, we employed a porcine model of chronic myocardial ischemia to examine, to our knowledge for the first time, whether a single application of FGF-2 is more effective as a protein or as DNA plasmid.

One week after creation of a severe LAD stenosis, the agents were injected intramyocardially across the ischemic area. Plasmid-driven protein synthesis could be proven after 2 days. Control animals were left untreated. The results were evaluated 3 months later.

The process of collateralization involves angiogenesis, which is the sprouting of new capillaries, and arteriogenesis, the development of arterial structures from small preexisting collateral vessels [12]. Arteriogenesis is considered to be more important than angiogenesis due to higher perfusion capacity of arterial vessels. In our model, untreated ischemia resulted in a significant reduction of capillaries, but not of arterioles. FGF-2 protein injections as well as plasmid injections caused an increase in the number of capillaries and arterioles compared to untreated ischemia.

Furthermore, after FGF-2 plasmid treatment a significantly higher number of arterioles were present even compared to normal myocardium. The reasons for this phenomenon are so far not clear. We did not observe related adverse effects in our study. A limitation of our assessment is that we only counted the vessels and did not measure their diameter. Therefore, the additional restructuring of small arterioles to larger vessels could have eluded our observations.

The results of our study are somewhat incongruous concerning regional myocardial blood flow and segmental myocardial shortening. Whereas regional contractility benefits from FGF-2 plasmid therapy at rest and under stress, a better perfusion of the ischemic area is observed only at rest. The improvement corresponds to the increase in the number of arterioles, which is strongest with gene therapy. However, the mere number of arteriolar structures does not reflect vessel quality. Therefore, the proper physiological response of newly-built vessels to stress cannot be predicted. This might explain the lack of flow reserve. The improvement of regional contractility, that was observed nevertheless, could be due to protective effects of FGF-2 on cardiomyocytes previously shown in vivo [13,14].

Systemic hemodynamics were examined by left ventricular stroke work index and dp/dt\text{max} at rest and under stress.

Fig. 5. Regional contractility assessed as SMS by ultrasonic crystals at rest (a); and under stress (b). SMS was recorded at rest before stenosis and at rest and under stress by 20\,\mu g/min per kg dobutamine after 1 week (baseline) and 3 months ischemia. Ischemia results in progressive deterioration of SMS. Only plasFGF-2 resuscitates regional contractility after 3 months at rest compared to the 1 week baseline and achieves significantly higher values under stress compared to the ischemic control. Values raised after three months are expressed as % of pre-ischemic SMS\textsuperscript{\pm}SD. * indicates a significant difference compared to baseline, the arrows express the direction of change. † shows a significant difference to the ischemic group.
conditions. There were improvements at some points following plasFGF-2 treatment, however, this was not a striking overall picture.

In the present study, angiogenesis and arteriogenesis were achieved by application of both FGF-2 protein and plasmid. Opinions differ as to whether angiogenesis can be caused by a mere needle puncture of the myocardium. Whereas Chu et al. [15] demonstrate angiogenesis, Horvath et al. [16] do not observe this effect. Both experiments were done in porcine models of myocardial ischemia in context with transmyocardial laser revascularization. In a clinical study, Pecher et al. [7] injected intramyocardially FGF-1 or, for control purposes, the heat denatured factor. The results proved the superiority of the active protein.

Our results are in line with previously reported effects of FGF-2 treatment. Following intramyocardial application in a porcine infarction model [17], arteriogenesis, but not angiogenesis were observed in the border zone of the infarction and even in normal myocardium. FGF-2 has also been employed recently in double-blind randomized clinical studies. Coronary ischemic disease was the subject of the FGF Initiating Revascularization Trial, wherein a single intracoronary infusion of recombinant FGF-2 achieved symptomatic improvement after three but not after 6 months but did not improve perfusion or exercise tolerance [18]. In contrast, intramyocardial injection of FGF-1 in combination with coronary artery bypass grafting rendered angiogenesis and an increase in the left ventricular ejection fraction [7]. In patients with peripheral arterial disease, intraarterial FGF-2 increased the calf blood flow [19]. However, it is difficult to compare results from different experimental and clinical settings, because ischemia, infarction, or even adding a surgical procedure, e.g. a coronary artery bypass grafting, provide different environments which could alter the influence of the growth factor.

In addition, one might speculate that different agents require different applications to function optimally. For instance, a protein might work best when delivered intracoronarily or intrapericardially, whereas gene therapy achieves better results when injected intramyocardially. The most effective application method might even differ for different growth factors depending on their cellular target, e.g. vascular endothelial cells or stromal cells.

Exogeneously delivered proteins are quickly eliminated in tissues and serum. Following intramyocardial injection of FGF-2, 5% of the injectate is retained for up to 3 days [20]. In contrast, gene expression from naked plasmid DNA under control of the CMV promoter was observed in cardiomyocytes for 8 days peaking on the first day after intramyocardial injection [21].

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Table 2
Systemic hemodynamics

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<thead>
<tr>
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<th>Rest (1 week)</th>
<th>Rest (3 months)</th>
<th>Stress (1 week)</th>
<th>Stress (3 months)</th>
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<tr>
<td></td>
<td>Mean</td>
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<td></td>
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<tr>
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<tr>
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</table>

* Global function as indicated by LVSWI and maximal increase of atrial pressure (dp/dt_{max}). Parameters were recorded using a Swan Ganz Catheter and an Arteria carotis Catheter at rest and under stress conditions brought on by dobutamine at a dose of 10 μg/min per kg. For details see Section 2. Values are given as mean and SD. The numbers of included animals are indicated. Under stress conditions, parameters could not be assessed from all animals due to their intraoperative condition. There were no improvements of LVSWI and dp/dt_{max} under stress. Significant differences are marked as follows: *P = 0.018 ischemia versus baseline, **P = 0.028 recFGF-2 versus baseline, ***P = 0.032 plasFGF-2 versus recFGF-2, ****P = 0.012 plasFGF-2 versus ischemia.
Although animal studies indicate that gene therapy employing single growth factors is able to promote unwanted vessel growth or atherosclerosis under certain conditions, a number of recent clinical trials have lead to the conclusion that gene therapy is a safe procedure implying a low risk of mortality. Additionally, no development of vascular malformations, neoplasms, atherosclerosis, or retinopathy was to be found in these human studies [22].

However, to avoid these potential problems – and to stimulate an effective collateralization more physiologically – several approaches of a multifactor-therapy are currently under investigation: combination of growth factors, injection of transcription factors like HIF, which control a series of genes, or application of stem/progenitor cells, which synthesize a set of factors.

In our experimental setting, FGF-2 plasmid application rendered better results than protein with regard to perfusion as well as regional contractility and systemic hemodynamics. They indicate, that FGF-2 plasmid therapy might be a more suitable tool for treatment of chronic myocardial ischemia than protein application.

References