

Induction of Neoangiogenesis in Ischemic Myocardium by Human Growth Factors

First Clinical Results of a New Treatment of Coronary Heart Disease

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Background—The present article is a report of our animal experiments and also of the first clinical results of a new treatment for coronary heart disease using the human growth factor FGF-I (basic fibroblast growth factor) to induce neoangiogenesis in the ischemic myocardium.

Methods and Results—FGF-I was obtained from strains of *Escherichia coli* by genetic engineering, then isolated and highly purified. Several series of animal experiments demonstrated the apathogenic action and neoangiogenic potency of this factor. After successful conclusion of the animal experiments, it was used clinically for the first time. FGF-I (0.01 mg/kg body weight) was injected close to the vessels after the completion of internal mammary artery (IMA)/left anterior descending coronary artery (LAD) anastomosis in 20 patients with three-vessel coronary disease. All the patients had additional peripheral stenoses of the LAD or one of its diagonal branches. Twelve weeks later, the IMA bypasses were selectively imaged by intra-arterial digital subtraction angiography and quantitatively evaluated. In all the animal experiments, the development of new vessels in the ischemic myocardium could be demonstrated angiographically. The formation of capillaries could also be demonstrated in humans and was found in all cases around the site of injection. A capillary network sprouting from the proximal part of the coronary artery could be shown to have bypassed the stenoses and rejoined the distal parts of the vessel.

Conclusions—We believe that the use of FGF-I for myocardial revascularization is in principle a new concept and that it may be particularly suitable for patients with additional peripheral stenoses that cannot be revascularized surgically. (*Circulation*. 1998;97:645-650.)

Key Words: growth substances ■ angiogenesis ■ coronary disease

For the cardiac surgeon who is attempting to treat CHD, the use of sections of autologous blood vessels as bypass material is subject to severe limitations. Autologous arterial conduits are in short supply, and segments of the saphenous vein do not remain patent for very long.^{1,2} Furthermore, "complete" revascularization is limited if diffuse coronary arteriosclerosis is present and extensive, especially if there are additional peripheral stenoses.

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In the search for alternative and/or additional treatment for improving the long-term prognosis, especially in diffuse CHD, attention has recently been directed toward natural angiogenesis.³⁻⁹ Growth factors, especially FGF-I, have recently become of major importance because they can induce angiogenesis.^{8,10-12}

Gimenez-Gallego et al¹³ succeeded in elucidating the biochemical structure of FGF-I in 1985. Jaye et al¹⁴ isolated human FGF-I from brain tissue in 1986. In 1991, Forough and coworkers¹⁵ successfully used the technique of gene transfer to introduce the information for expressing human FGF-I into apathogenic *Escherichia coli*.

Our aim was to evaluate the information currently available on the biological effect of angiogenetic growth factors in animals and, if appropriate, to use human growth factor for the

treatment of CHD. This involved (1) the production of human growth factor by genetic engineering, followed by its isolation, characterization, and purification; (2) using animal experiments to establish its angiogenetic potency and to exclude any possible pathogenic effect; and (3) using FGF-I clinically as an adjunct to coronary surgery and to demonstrate neoangiogenesis in the ischemic human myocardium.

Methods

Production and Purification of FGF-I

The production and purification of human FGF-I is a biochemically elaborate technique. The individual experimental steps have been reported elsewhere.^{4,7}

Genetic engineering was used to produce human FGF-I from apathogenic strains of *E coli*, a plasmid containing the genetic information being introduced into the microorganisms.¹⁵ These were kindly provided by Prof T. Maciag (Laboratory of Molecular Biology, American Red Cross, Rockville, Md). After production, FGF-I was eluted by heparin sepharose column chromatography, and several elution fractions were collected and purified by dialysis. Positive protein elution fractions were identified in the BIO-RAD assay⁷ by SDS-PAGE,¹⁶ and the biochemical isolation of FGF-I was confirmed by the Western blot method.¹⁷ Further purification was obtained by HPLC.¹⁸ The factors were lyophilized and stored at -32°C and diluted to 1 mL with NaCl solution containing 500 IU of heparin.

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Selected Abbreviations and Acronyms

CHD	= coronary heart disease
EDP	= electronic data processing
FGF	= basic fibroblast growth factor
HPLC	= high-pressure liquid chromatography
IMA	= internal mammary artery
LAD	= left anterior descending coronary artery

Chorioallantoic Membrane Assay

This established method, which provides a direct demonstration of the effect of growth factors on living tissue, was used to investigate the angiogenic effect of FGF-I.^{19,20} The growth of the allantoic systems can be directly observed by light microscopy. After incubation of 20 fertilized hen eggs for 13 days, the growth factor was applied to the membrane and covered with tissue culture coverslips. Four days later, the membrane was examined under the light microscope and directly compared with controls untreated with FGF-I or treated with heat-denatured FGF-I (70°C for 3 minutes).

Exclusion of the Pyrogenicity of FGF-I

Varying concentrations of FGF-I (0.01, 0.5, or 1.0 mg/kg body weight) were injected subcutaneously, intramuscularly, or intravenously into 27 New Zealand White rabbits, the solvent alone being used for an additional 13 controls. Thereafter, the rectal temperature was taken every half hour for 3 hours, hourly for the rest of the day, and every 8 hours for 12 days. A daily white cell count was also repeated for 12 days (see "Results"). In addition to this, the erythrocyte sedimentation rate and the C-reactive protein values were determined on the 3rd, 6th, 9th, and 12th days after the injection.

Confirmation of the Angiogenic Potency of FGF-I in Animal Experiments

Supplementary to our earlier experiments,^{4,7} the effect of FGF-I was also investigated in the ischemic hearts of inbred Lewis rats (a total of 275 animals, including 125 controls treated with heat-denatured FGF-I, 70°C for 3 minutes). The pericardium was opened via the abdominal wall and diaphragm, and two titanium clips were inserted at the apex of the left ventricle to induce myocardial ischemia. Growth factor (mean concentration of 10 µg) was then injected locally into the site. The coronary vessel system was imaged by aortic root angiography after 12 weeks and, finally, a specimen from the same myocardial region was evaluated histologically.

Clinical Use of FGF-I in Patients With CHD

This study was approved by the Medical Research Commission at the Phillips University of Marburg on August 10, 1993 (No. 47/93). This is the usual ethics commission for our hospital. Twenty patients without any history of infarction or cardiac surgery (14 men and 6 women; minimum age, 50 years) were subjected to an elective bypass operation for multivessel coronary heart disease. The growth factor was applied directly during the operation. As a control group, 20 patients who underwent the same procedure were given heat-denatured FGF-I (70°C for 3 minutes). The choice of treatment was completely random, the names being placed in sealed envelopes and selected in a blinded manner.

The details, nature, and aims of this procedure were explained beforehand to every patient who underwent the operation. In all cases, we received their fully informed consent. Both groups of patients were closely comparable with regard to clinical symptoms, accompanying disorders, cardiovascular risk factors, ventricular function, sex, and age. A comparable coronary morphology was found in both groups.

All patients had a further stenosis in the distal third of the LAD or at the origin of one of its branches in addition to a severe proximal stenosis. The mean ejection fraction of the left ventricle for all patients was 50%. The operative procedure for coronary revascularization with autologous grafts (an average per patient of 2 to 3 venous bypasses and 1 from the left IMA) was routinely performed. FGF-I (mean concen-

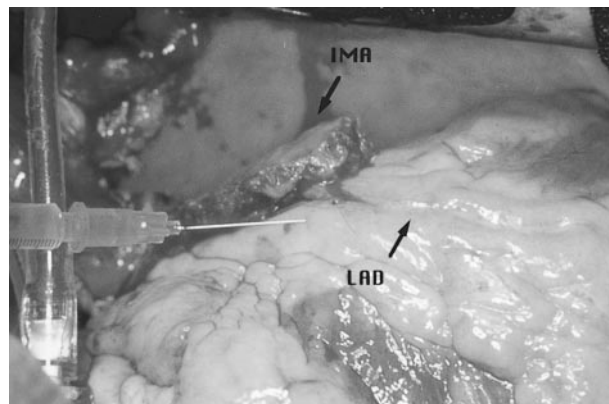


Figure 1. Intraoperative administration of growth factor.

tration, 0.01 mg/kg body weight) was injected into the myocardium, distal to the IMA/LAD anastomosis and close to the LAD, during the maintenance of the extracorporeal circulation and after completion of the distal anastomoses (Fig 1). In the control group, heat-denatured FGF-I was substituted for FGF-I. After 12 weeks, the IMA bypasses of all the patients were imaged selectively by transfemoral, intra-arterial, and digital subtraction angiography.

Angiograms obtained in this way were evaluated by means of EDP-assisted digital gray-value analysis, a universally recognized and well-established technique for demonstrating capillary neoangiogenesis.²¹⁻²⁶ Sites of interest both with and without FGF-I (meaning heat-denatured FGF-I) were selected in the vessels filled with contrast medium and in regions of the myocardium distal to the IMA/LAD anastomosis. One hundred pixels were selected from each site of interest and analyzed digitally. Complete blackening of the x-ray films was rated with a gray value of 150, and areas without blackening of the film were allotted a zero value. During the first 5 postoperative days, separate laboratory checks in addition to the routine postoperative follow-up procedures were made twice daily, and the temperature checked three times a day.

Results

After separation, purification, and stabilization, we were able to isolate human FGF-I in all 40 bacterial cultures and demonstrate its high degree of purity. Fig 2 shows an HPLC profile of the growth factor after routine purification. The peak values at the beginning and end of the profile represent impurities that could be identified as *E coli* proteins. FGF-I could be further separated by fractionated collection, and the control HPLC (Fig 3) merely shows the peak value of this fraction on an otherwise even baseline.

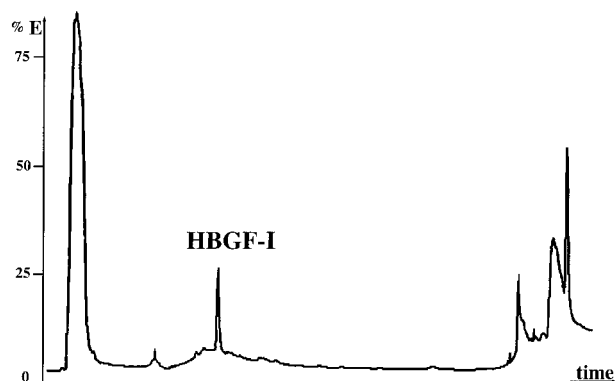


Figure 2. HPLC profile before high purification. HBGF-I indicates human FGF-I; %E, extinction.

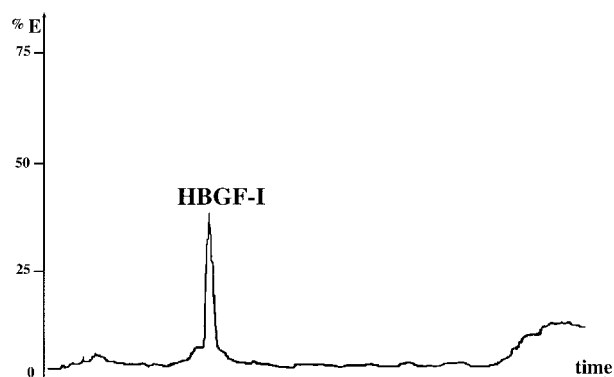


Figure 3. HPLC profile after high purification. HBGF-I indicates human FGF-I; %E, extinction.

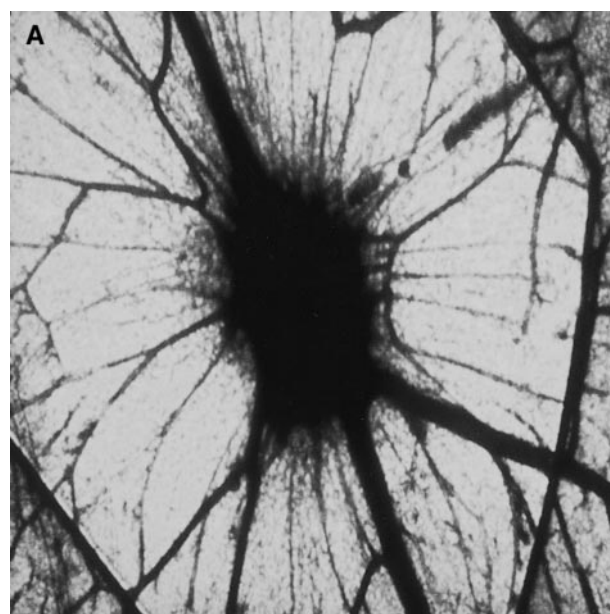
In the chorioallantoic membrane assay, the angiogenic potency of FGF-I could be demonstrated *in vivo*. As early as 4 days after application of the factor, the vascular structure of the membrane was completely altered. Emanating radially from the site of application, an unequivocal growth of new vessels had grown out into the periphery (Fig 4A). These structures were completely absent from the control group, and a normally developed reticular vascular pattern could be discerned (Fig 4B).

Pyrogenic effects of the human growth factor produced in this way could be definitively ruled out in the animal model. There was no significant rise of body temperature when checked at short intervals and no trace of an inflammatory reaction in comparison with the control group ($n=13$) in any of the 27 test animals during the period of observation. This result was independent of the concentration and the route of administration (intravenous, subcutaneous, or intramuscular) of the factor.

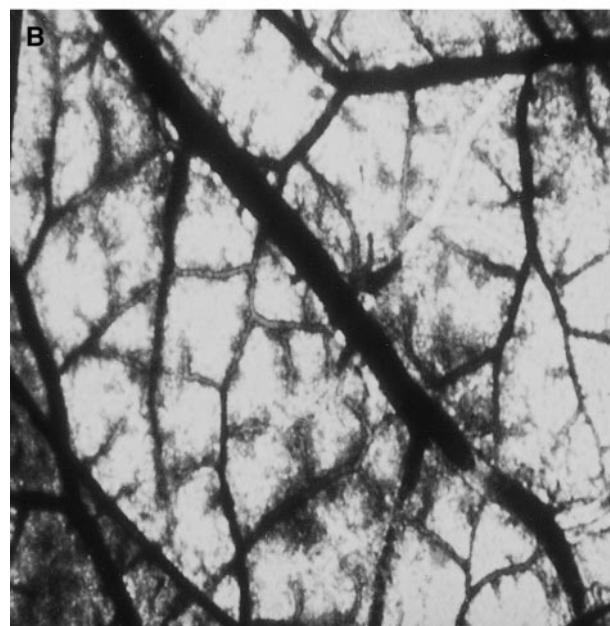
Earlier investigations into the application of FGF-I to the nonischemic rat heart made it possible to demonstrate neoangiogenesis both histologically and angiographically after 9 weeks in 11 of 12 test animals after the implantation of a tissue bridge pretreated with growth factor between the heart and thoracic aorta. In the control group without FGF-I ($n=6$), no signs of induced neoangiogenesis could be found.^{4,7}

Unequivocal proof of induced neoangiogenesis was also found in the ischemic rat heart. In the test animals, in which myocardial ischemia had previously been induced with titanium clips and growth factor had subsequently been injected into the myocardium, a manifest accumulation of contrast medium was shown by aortic angiography at the site of the FGF-I injection 12 weeks later (Fig 5A), whereas such an accumulation of contrast medium did not appear in any of the control animals (Fig 5B). Histological examination of the myocardium revealed a threefold increase in the capillary density per square millimeter around the site of the FGF-I injection.

When the growth factor FGF-I was used clinically for the first time on the human heart, neoangiogenesis together with the development of a normal vascular appearance could be demonstrated angiographically, exactly as in the earlier animal experiments.^{4,7} Selective imaging of the IMA bypasses by intra-arterial digital subtraction angiography confirmed the following result in all 20 patients: at the site of injection and in the distal areas supplied by the LAD, a pronounced accumulation of contrast



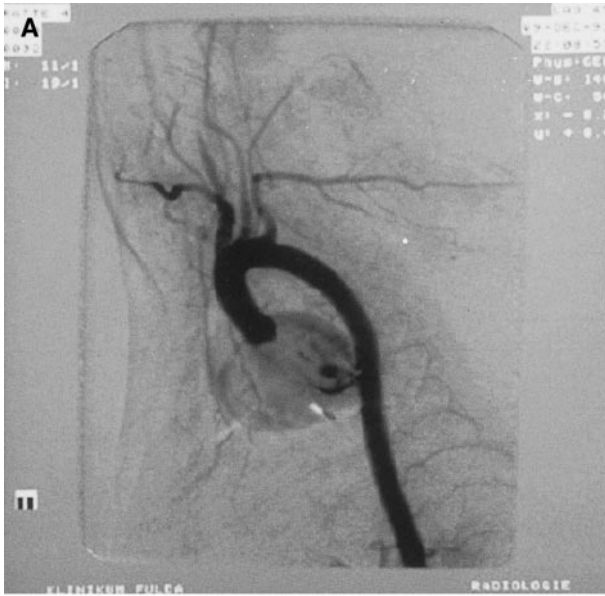
10 ng HBGF-I



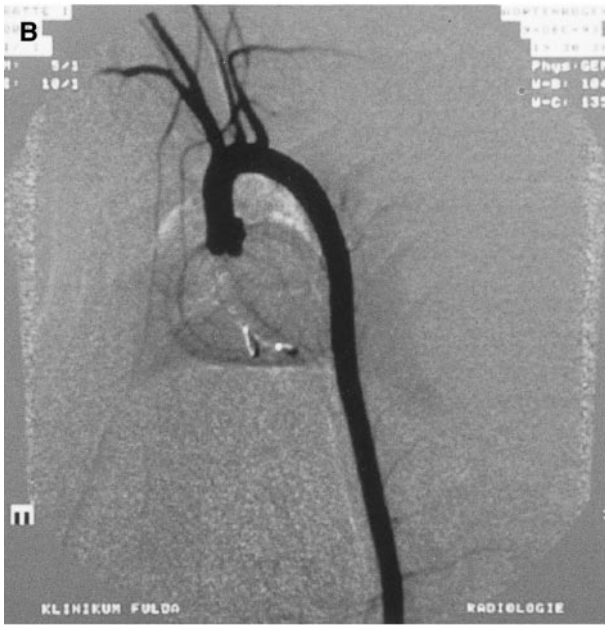
without HBGF-I

Figure 4. A, Chorioallantoic membrane assay with application of the growth factor. B, Chorioallantoic membrane assay of the control group. HBGF-I indicates human FGF-I.

medium extended peripherally around the artery for ≈ 3 to 4 cm, distal to the IMA/LAD anastomosis (Fig 6A). In the control angiograms of patients to whom only heat-denatured FGF-I had been given, the IMA/LAD anastomosis was also recognizable, but the accumulation of contrast medium described above was absent (Fig 6B). The angiograms of both the treated and control groups were recorded at a rate of four images per second, and these show



10 µgHBGF-I



without HBGF-I

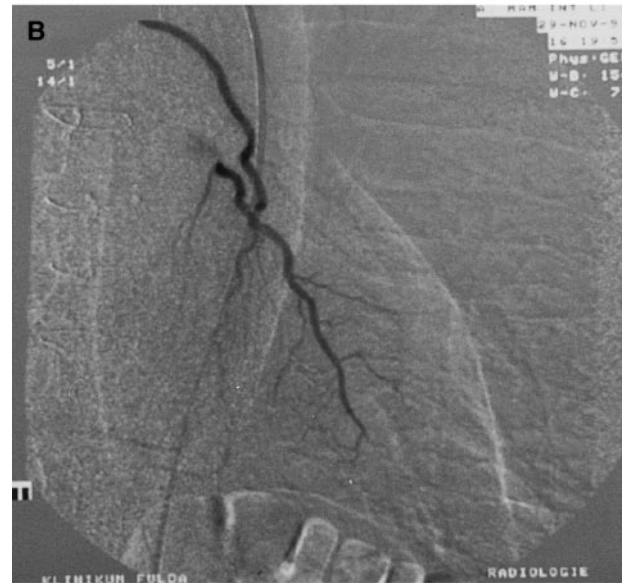
Figure 5. A, Administration of the growth factor in ischemic rat heart with a clearly discernible accumulation of contrast medium at the site of injection. B, No discernible accumulation of contrast medium in the control group. HBGF-I indicates human FGF-I.

comparable distances between the beginning of the injection and visualization of the medium.

At the site of injection of the FGF-I, a capillary network could be seen sprouting out from the coronary artery into the myocardium. This enabled retrograde imaging of a stenosed diagonal branch to be performed (Fig 7A). Such “neocapillary vessels” can also provide a collateral circulation around additional distal stenoses of the LAD (Fig 7B) and bring about



10 µg/kg HBGF-I

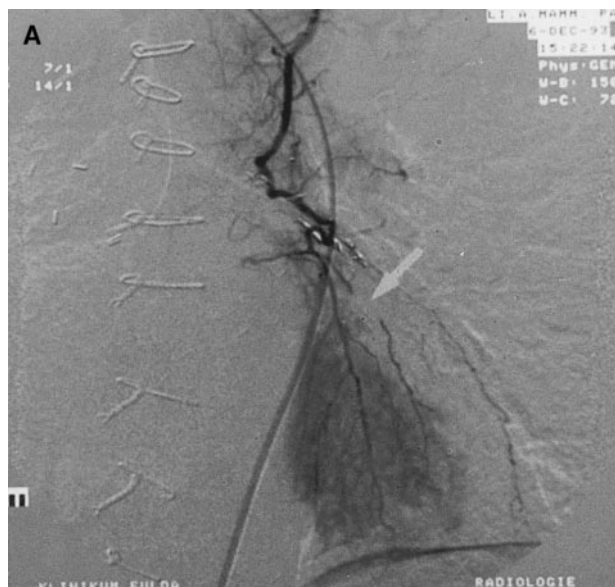


without HBGF-I

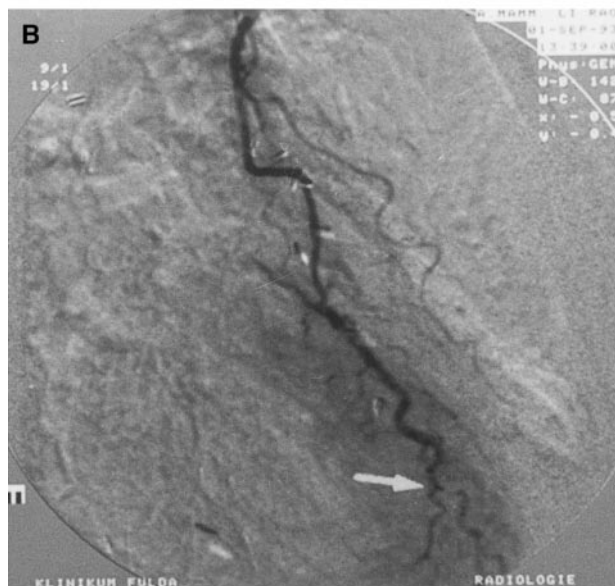
Figure 6. A, Angiography after injection of the growth factor into the human heart shows a pronounced accumulation of contrast medium compared with the control group. B, Angiography in the control group does not show any increased accumulation of contrast medium around the IMA/LAD anastomosis. HBGF-I indicates human FGF-I.

retrograde filling of a short segment of the artery distal to the stenosis. In none of the angiograms of the treated patients taken 12 weeks after the operation were any new stenoses of the LAD detectable.

The results of EDP-assisted digital gray value analysis for quantification of the neoangiogenesis (Fig 8) gave a mean gray value of 124 for the vessels. The control myocardium reached



10 µg/kg HBGF-I



10 µg/kg HBGF-I

Figure 7. A, Collateralization of stenoses (arrow): a diagonal branch occluded just distal to its origin is filled through the newly grown capillaries. B, Collateralization of stenoses (arrow) by newly grown capillaries: the peripherally stenosed LAD is filled through these vessels. HBGF-I indicates human FGF-I.

a gray value of only 20, and that of the myocardium injected with FGF-I gave a value of 59 (Fig 8).

Discussion

Normal capillaries have a cell population with a low turnover rate of months or years. On occasion, however, a high turnover rate of this cell population is possible even under physiological

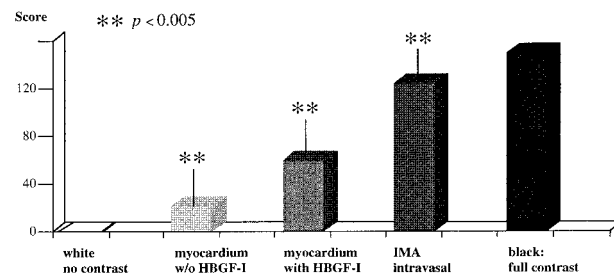


Figure 8. Quantitative gray value analysis of contrast medium accumulation in the angiography shows a twofold to threefold increase in the local blood flow at the site of injection. HBGF-I indicates human FGF-I.

conditions, and this naturally leads to the rapid growth of new capillaries and other blood vessels. Such a physiological process occurs in the development of the placenta, in fetal growth, and in wound healing, as well in the formation of collaterals in response to tissue ischemia. “Angiogenic growth factors,” which are biochemically polypeptides, are essential for such processes as capillary growth or neovascularization. These growth factors (for instance, the human heparin-binding FGF-I) bring about their effect by significantly increasing cell proliferation, differentiation, and migration via a high-affinity receptor system on the surfaces of the endothelial cells.^{8,10–12}

During the last few years, several working groups have been able to establish indications for the effective use of growth factors to improve blood flow in the presence of tissue ischemia in animal experiments.^{3,9,27} Yanagisawa-Miwa et al⁹ succeeded in demonstrating a significant collateralization together with reduction in the size of the infarct after intracoronary administration of growth factor in rabbits. Baffour et al³ also observed a significant formation of collaterals in ischemic extremities after growth factor administration in animals. Albes et al²⁷ produced a distinct improvement in the blood flow in ischemic tracheal segments implanted subcutaneously in rabbits by injecting growth factor-enriched fibrin glue locally.

After growth factor was injected into the ischemic rat heart,^{4,7} we were able to observe induced neovascularization and confirm it angiographically. We were also able to prove histologically that this neovascularization brings about the development of new vascular structures with a three-layered vessel wall. Angiographic imaging confirmed that these are anatomically normal capillaries and other blood vessels.

The production of human FGF-I by our molecular biological method has proved to be a complex but readily reproducible procedure. From the bacterial cultures, we are able to isolate the factor as a pure substance in sufficient quantities. By *in vitro* assay and as a result of extensive animal experiments, we were able to exclude the possible pyrogenic effects of FGF-I.

In earlier animal experiments,⁴ we were able to demonstrate the proliferative and mitogenic effects of the growth factor on human saphenous vein endothelial cells. Endothelial cell cultures with added growth factor induced a confluent monolayer after only 5 to 9 days, whereas the monolayer was not complete before 7 to 11 days in the control group. In addition to determining the total cell count with a cell counter, we also confirmed this result by analyzing the rate of DNA synthesis by measuring the incorporation of ³H-thymidine into the endothelial cell nuclei using the

method of Klagsbrun and Shing.²⁸ The cell proliferative potency of FGF-I could be further intensified by adding heparin, a glycosaminoglycan protecting the growth factor from inactivation by cellular enzymes and from heat and chemical denaturation.²⁹

On the basis of these *in vitro* and *in vivo* experiments, we established for the first time the efficacy of FGF-I for the treatment of CHD, and were able to demonstrate that it can induce neovascularization *in situ* in the ischemic human heart. This possibility has been widely discussed for many years but never before attempted.

A dense capillary network appeared around the site of injection of the factor in the myocardium of all our treated patients. This capillary network is a true *de novo* vascular system. Emerging from the proximal segment of the LAD, it sprouts out into the surrounding myocardium, bringing about a twofold to threefold increase in the local blood supply through these newly formed functional vessels. We were able to use the recognized physiological effects of FGF-I (as they occur in the repair mechanism of wound healing or in collateralization of ischemic tissue) to induce neovascularization in the human ischemic heart.

We also consider that administration of FGF-I (produced in this way by genetic engineering), combined with operative myocardial revascularization, may well be an especially appropriate treatment for patients with additional peripheral stenoses that cannot be treated surgically.

In our opinion, neovascularization induced by FGF-I opens up new possibilities for the treatment of ischemic myocardial disease. Furthermore, it could become a new therapeutic concept in the management of diffuse CHD after alternative methods of administration have also been developed. This method of inducing neovascularization is also conceivable as a therapeutic option in other regions of the cardiovascular system in which arterial occlusion has led to ischemia.³⁰ However, before any such possibilities are realized, many more clinical investigations will have to be performed.

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