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Original Article

The effects of basic fibroblast growth factor in an animal model of acute mechanically induced right ventricular hypertrophy

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Abstract *Objective:* To evaluate the effect of a continuous infusion of basic fibroblast growth factor on the adaptive potential of the right ventricular myocardium after 30 days of mechanically induced overload in rats. *Materials and methods:* We banded the pulmonary trunk, so as to increase the systolic workload of the right ventricle, in six Lewis/HanHsd rats at the age of 11 weeks, using six adult rats as controls. The six adult rats were also banded and received an additional continuous infusion of basic fibroblastic growth factor, using six rats with a continuous infusion of basic fibroblastic growth factor only as controls. We analysed the functional adaptation and structural changes of the right ventricular myocardium, blood vessels, and interstitial tissue 30 days after the increased afterload. *Results:* The pulmonary artery banding induced an increase in the right ventricular free wall thickness of banded rats when compared with controls, which was mainly justified by an increase in cardiomyocyte area and in the percentage of extracellular fibrosis. The infusion of basic fibroblastic growth factor promotes a more extensive capillary network in banded rats ($p < 0.001$), which modulates the compensatory response of the right ventricle, promoting the hypertrophy of contractile elements and limiting the areas in which fibrosis develops ($p < 0.001$). *Conclusions:* The subcutaneous infusion with osmotic pumps was a valid and reproducible method of delivering basic fibroblast growth factor to heart tissue. This infusion contributed to better preserve the right ventricular capillary network, hampering the development of interstitial fibrosis.

Keywords: Cardiac failure; ventricular remodelling; congenital defects; extracellular matrix

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Cardiac hypertrophy is an adaptive response to a sustained increase in ventricular workload. Its effect is to decrease the ventricular wall stress, and thus preserve the cardiac function. However, if pressure overload remains unrelieved, a progressive ventricular dilatation occurs with an increase in wall stress, followed by a progressive deterioration of myocardial function.[1–3] Under conditions of physiologic hypertrophy, the coronary micro-vascular grows parallel to the degree of cardiac myocyte growth, between childhood and young adulthood. On the contrary, during pathologic hypertrophy, this tight relationship appears to be lost.[4] Several patients with congenital heart disease have the morphologic right ventricle as the pump to the systemic circulation. Long-term follow-up of these patients shows progressive development of right ventricular hypertrophy and failure in up to one-tenth of patients for every 10 years.[5–6]

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With this concept in mind, we have described previously an experimental animal model of acute increased afterload of the right ventricle by banding the pulmonary artery trunk, showing that the right ventricle seems to have a maladaptive response under systemic pressure conditions with a significant increase in interstitial fibrosis together with a decreased micro-vascular density.[7]

Consequently, we hypothesised that by increasing the vascular support of the right ventricular myocardium, during mechanically induced ventricular hypertrophy, we could have modified the mechanism of myocardial adaptation hampering the development of interstitial fibrosis.

On the basis of that, we sought to evaluate, in an animal model, the effects of a continuous subcutaneous infusion of basic fibroblast growth factor on the right ventricular myocardium, after 30 days of mechanical-induced right ventricular overload.

Material and methods

Animals and experimental groups

A total of 24 11-week-old male inbred Lewis/HanHsd rats from Harlan Laboratories (San Pietro al Natisone, Udine, Italy) were randomly divided into four experimental groups: rats that underwent banding of the pulmonary artery (n = 6); rats that underwent pulmonary artery bandage plus a continuous infusion of basic fibroblast growth factor (n = 6); control rats (n = 6); and control rats plus a continuous infusion of basic fibroblast growth factor (n = 6).

All animals were kept in conventional facilities with free access to food and water. Adequate care for their health and well-being was provided. These studies were performed in accordance with the guidelines and regulations set forth by the local Ethics Committee and according to the Italian Law on the use of experimental animals.

The operative procedures were carried out as described previously, and the pulmonary artery trunk was banded proximal to its bifurcation using a 4.0 silk suture to produce a 60% decrease in its diameter.[9] A long-acting antibiotic, 60 milligrams per kilogram of oxytetracycline, was administered subcutaneously to the rat for infection prophylaxis. The animal was placed under a heating lamp and monitored visually until it was fully awake, which usually took up to 5 minutes after suspending anaesthesia. The rat was then left to recover at 22–24°C room temperature, together with other animals, with immediate, unrestricted access to food and water. Peri-operative pain was controlled with intramuscular tramadol hydrochloride (5 milligrams per kilogram), every 12 hours, for the first 3 post-operative days. The pulmonary artery banding was kept in place for 30 days. After the first post-operative week, when they were closely monitored, rats were subsequently controlled and weighed three times a week.

Osmotic pump implantation

Recombinant human basic fibroblast growth factor (Invitrogen Inc., Carlsbad, California, United States of America) was delivered by means of a subcutaneous osmotic pump (Mini-Osmotic ALZET® pump model 2004, Durect Corporation, Cupertino, California, United States of America). The osmotic pumps were filled up in a sterile manner, with a solution of 600 nanograms of basic fibroblast growth factor diluted to a volume of 200 millilitres with 0.1% bovine serum albumin in phosphate-buffered saline solution filtered through a 0.45 millimetres Millipore filter. The resultant dose of basic fibroblast growth factor was 18 nanograms per day and the delivery rate was of 0.25 microlitres per hour. On post-operative day 2, the osmotic pump was implanted subcutaneously in the inter-scapular area of the rats, and left in situ for the rest of the experiment. The brief surgical procedure was performed under anaesthesia with sevoflurane 1.5–2% and oxygen, supplemented with 5 milligrams per kilogram of intramuscular tramadol hydrochloride.

Functional tests

All rats that survived the banding procedure were monitored by echocardiogram on post-operative day 30, using a Hewlett-Packard Sonos 5500 echocardiography apparatus equipped with a 12.5 Megahertz probe (Hewlett Packard, Palo Alto, California, United States of America). The gradient across the band was measured and an indirect measure of the right ventricular pressure was assumed for testing the accuracy of the induced right ventricular overload.

Preparation of samples for morphological analysis

On post-operative day 30, the experimental endpoint, all animals were euthanised with an overdose of zolazepam tiletamine (Virbac Srl, Milan, Italy), followed by 0.5 millilitre of intrapulmonary Tanax® (Intervet Italia Srl, Milan, Italy); 1 millilitre contains: embutramide 200 milligrams, mebenzonium 50 milligrams, and tetracaine chlorydrate 5 milligrams. During necroscopy, they were inspected for possible onset of hydrothorax and/or ascites. Body weight and wet heart weight were also measured. Hearts were quickly perfused with an antegrade cold cardioplegic solution to obtain a diastolic arrest, were harvested, and washed with phosphate-buffered saline. The liver, the foregut, one kidney, and a blood sample were also obtained, but not analysed in this study. All hearts were

sectioned in a short axis-view plane 5 millimetres distant from the apex of the heart. The osmotic pumps were also harvested and weighed.

Histology and immunohistochemistry

The hearts were placed in an optimal cutting temperature compound (Tissue Tek, Miles Inc., Elkhart, Indiana, United States of America) and immediately frozen in liquid nitrogen and stored at −80°C.

Transverse cryosections (10 micron) were processed for haematoxylin–eosin staining to determine the ventricular wall and septal thickness. Measurements were calculated using the average of 10 fields for each sample. Cardiomyocyte area was calculated as the median of 80 cells sectioned equatorially. The percentage of fibrosis was evaluated on Heidenhain staining modified Azan–Mallory trichrome staining. Morphological and morphometric measurements were carried out using a computerised image analysis system (Leica Qwin system, Wetzlar, Germany).

Immunohistochemistry

Capillaries were identified by immunohistochemistry using an anti-von Willebrand factor polyclonal antibody (Chemicon, Temecula, California, United States of America) and anti-rabbit immunoglobulin G coupled with fluorescein isothiocyanate as secondary antibody (Dako, Glostrup, Denmark). Primary antibodies were applied to freshly cut, unfixed cryosections (8 micrometres) and incubated at 37°C for 30 minutes. After rinsing with phosphate-buffered saline solution, the sections were treated with goat anti-rabbit immunoglobulin G coupled with horseradish peroxidase. Bound immunoglobulin G was revealed by incubation in amino-ethyl-carbazole solution. The controls for indirect immunohistochemistry were rabbit non-immune immunoglobulin G rather than primary antibody, and the secondary antibody alone. Subsequent computerised image analysis was carried out to quantify capillary density defined as the total number of capillaries per square micron within 10 different randomly chosen fields in the right ventricle. Sections were observed using a Leica DMRE microscope equipped with a DC300-digital camera and a dedicated Qwin Software (Spectraservice, New York, United States of America).

Statistics

Median value and range were used for descriptive statistics. Owing to the small number of cases in every group, comparison among groups was carried out by non-parametric tests: Wilcoxon signed-rank test – comparison between two groups – or Kruskal–Wallis test – comparison among four groups. Data were analysed using SAS software, release 9.2 (SAS Institute Inc., SAS Stat 9.1®, Cary, North Carolina, United States of America), and p-values below 0.05 were considered significant.

Results

All rats survived for the overall period of the study (30 days). At the time of euthanasia, no signs of right-sided cardiac failure such as hydrothorax, hepatic cirrhosis, or ascites were detected in any of the rats. A total of 23 rats were processed for our analysis. However, one rat in the pulmonary artery banded group was not analysed because the banding was too tight, with a gradient of 70 millimetres of mercury across the band (Table 1).

Functional study – echocardiography

The pressure gradient across the banded pulmonary trunk was comparable between rats that underwent pulmonary artery banding and rats that had pulmonary artery banding plus basic fibroblast growth factor infusion (40 millimetres of mercury,

Table 1. Morphometric analysis.

	PAB rats (n = 5)	PAB rats + bFGF (n = 6)	Controls (n = 6)	Controls + bFGF (n = 6)	p-value
Median PAB gradient, mmHg (range)	40 (35–50)	40 (35–46)	–	–	0.58
Median pre-operative BW g (range)	334 (330–345)	325 (320–334)	326 (323–330)	329.5 (326–366)	0.06
Median post-operative BW g (range)	380 (360–425)	381 (367–396)	388 (378–394)	388 (365–400)	0.95
Median heart weight, g (range)	1.05 (0.93–1.15)	0.96 (0.84–1.12)	0.92 (0.88–0.93)	0.80 (0.75–0.93)	0.005
Median RV-free wall thickness, μm (range)	1.65 (1.43–1.78)	1.56 (1.09–1.67)	0.96 (0.55–1.14)	1.04 (0.57–1.32)	0.002
Median % interstitial fibrosis, % (range)	0.71 (0.66–0.85)	0.37 (0.30–0.66)	0.05 (0.04–0.07)	0.04 (0.04–0.08)	0.0003
Median RV myocyte area, μm^2 (range)	91.0 (82.6–107.1)	93.7 (70.0–96.4)	40.4 (34.3–42.3)	43.8 (35.6–45.0)	0.0006
Median RV capillary/100 μm^2 (range)	146 (135–163)	168 (162–173)	188 (186–200)	208 (206–210)	0.0001
Median LV capillary/100 μm^2 (range)	396 (373–443)	380 (312–452)	348 (323–373)	391 (338–393)	0.14

bFGF = basic fibroblast growth factor; BW = body weight; IQR = inter-quartile range; LV = left ventricle; PAB = pulmonary artery banding; RV = right ventricle

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Table 2. Comparison between two adjacent categories (see Table 1).

	p-values			
	PAB rats versus PAB rats + bFGF	**PAB rats versus Controls**	**PAB rats + bFGF versus Controls + bFGF**	**Controls versus Controls + bFGF**
Median PAB gradient (mmHg)	0.59	–	–	–
Median pre-operative BW (g)	0.08	0.06	0.25	0.19
Median post-operative BW (g)	0.93	1	0.69	1
Median heart weight (g)	0.43	0.031	0.04	0.09
Median RV-free wall thickness (μm)	0.24	0.024	0.05	0.39
Median % interstitial fibrosis (%)	0.028	0.024	0.016	0.80
Median RV myocyte area (μm^2)	0.78	0.024	0.017	0.15
Median RV capillary/100 μm^2	**0.033**	0.024	0.017	0.016
Median LV capillary/100 μm^2	0.65	0.031	0.81	0.15

bFGF = basic fibroblastic growth factor; BW = body weight; IQR = inter-quartile range; LV = left ventricle; PAB = pulmonary artery banding; RV = right ventricle

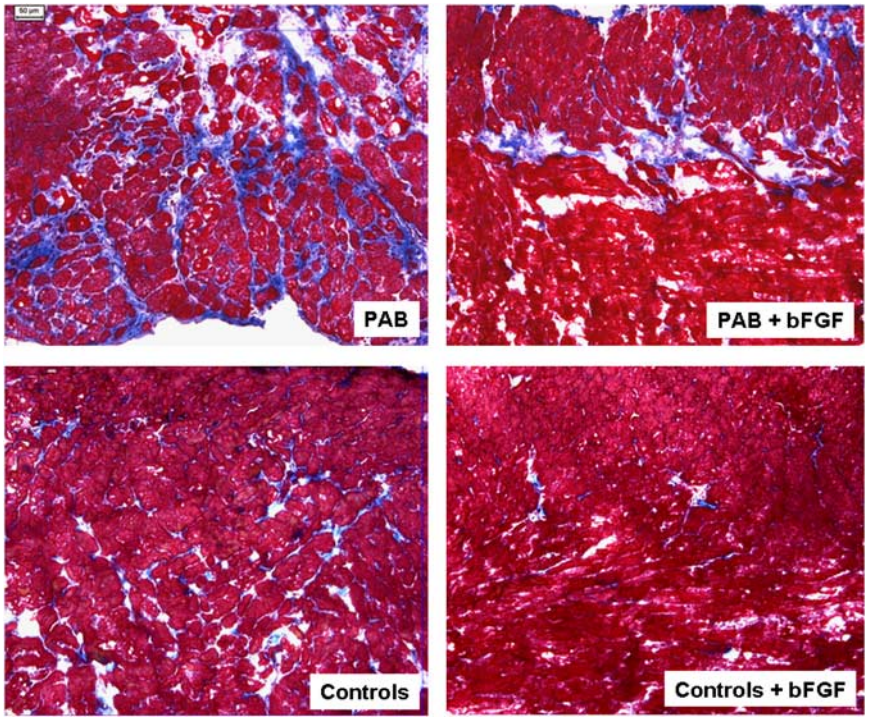

Figure 1.
Azan–Mallory trichrome Heidenhain modified staining of cardiac sections from the systemic morphologically right ventricle taken from a banded rat (PAB) and banded rats plus basic fibroblastic growth factor (PAB + bFGF), compared with controls (Controls) and controls plus basic fibroblastic growth factor (Controls + bFGF). Note the significant increase in extracellular fibrosis in PAB rats. PAB, pulmonary artery banded; bFGF, basic fibroblastic growth factor.

range 40–50 millimetres of mercury versus 40 millimetres of mercury, range 37–44 millimetres of mercury; p = ns; Table 1).

Morphometric study

Heart weight, right ventricular free wall thickness, cardiomyocyte transverse area, percentage of myocardial fibrosis, and capillary density are listed in Table 1.

Wet heart weight and the right ventricular free wall thickness were found to be increased in banded rats – with or without basic fibroblastic growth factor – when compared with their controls; however, the increase was significant only in banded rats when compared with controls (p-value of 0.05 and 0.04, respectively; Table 2). The increase in the right ventricular free wall thickness was likely to be due to a mixed increase in cardiomyocyte area

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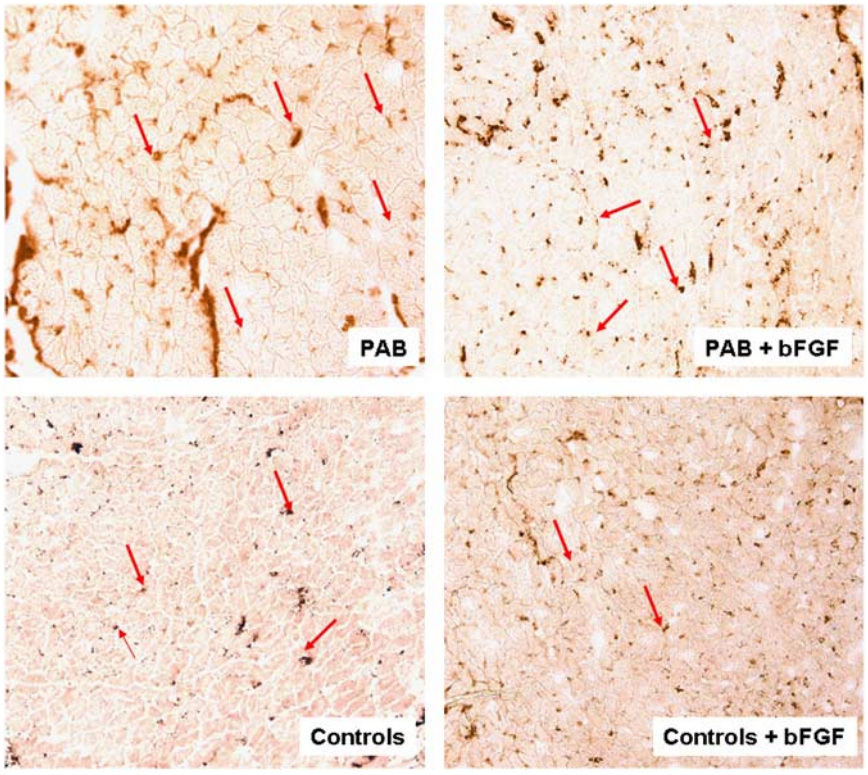

Figure 2.
Anti-von Willebrand factor polyclonal antibody staining of cardiac sections from the systemic morphologically right ventricle taken from a banded rat (PAB) and banded rats plus basic fibroblastic growth factor (PAB + bFGF), compared with controls (Controls) and controls plus basic fibroblastic growth factor (Controls + bFGF). Note the significant increase in the capillary density in PAB + bFGF and in Controls + bFGF rats. PAB, pulmonary artery banded; bFGF, basic fibroblastic growth factor.

(p-value of 0.001) and in the percentage of extracellular myocardial fibrosis (p-value of 0.002). The latter was found to be significantly lower in banded rats with basic fibroblast growth factor infusion when compared with banded rats without basic fibroblast growth factor infusion (p-value of 0.01); nonetheless, it was found to be still higher than in both control groups (Fig 1; Tables 1 and 2).

The right ventricular capillary density was found to be decreased in banded rats without basic fibroblast growth factor infusion when compared with controls without basic fibroblast growth factor infusion (p-value of 0.03). The infusion of basic fibroblastic growth factor seemed to preserve the capillary vascular network in banded rats when compared with the one of rats that underwent pulmonary artery banding alone; however, the number of capillaries for square micron was still lower than that in control groups (Fig 2; Tables 1 and 2).

It is of note that there were no significant differences in left ventricular capillary density between banded rats and controls in both groups with or without basic fibroblast growth factor infusion, possibly because of the lack of pro-adjuvant hypertrophic stimulus on the left side of the heart.

Discussion

During the development of hypertrophy, several adaptive changes occur, which include the multiplication of sarcomeres,[8,9] leading to an increase in cellular diameter, the switch to immature isoforms of contractile proteins,[10,11] and the greater dependence on trans-sarcolemmal calcium influx for excitation–contraction coupling.[12] On the other hand, the ventricular pressure overload activates and the increased wall stress activate the Renin–Angiotensin–Aldosteron system with consequent stimulation of fibroblast's division with the result of an increased collagen deposit. Fibrosis is irreversible and its increase hampers the contractile (systolic) function of the cardiomyocytes.[13–15]

It is also well known that in pathological ventricular hypertrophy the micro-vascular density does not grow parallel to the increase of cardiomyocyte diameter; the mismatch which developed between the number of capillaries and cardiomyocytes leads to an increase in diffusion distance and to a limited supply of oxygen and nutrients.[16–21]

Several studies have been conducted for analysing the morphological and physiological causes of

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systemic right ventricular failure.[4–6] Even if an impaired myocardial flow reserve of the right ventricle has been advocated as the possible cause for myocardial failure, the definitive and true history of the right ventricle supporting a systemic pressure in humans is not as yet known, and very few is its structural adaptation to the different workload.[7–23]

Our experimental study derivates from a previously reported and reproducible model of acute increased afterload of the morphologically low-pressure right ventricle by banding the pulmonary artery.[7] Previous results showed a significantly increased right ventricular wall thickness and weight, predominantly because of the hypertrophy of the cardiomyocytes and the development of interstitial fibrosis, which can be interpreted as a maladaptive response to stress due to the decreased capillary/myocyte ratio.

According to the results of the current study, we demonstrated that an increased right ventricle vasculature played a role in the adaptive response of the myocardium to acute pressure overload. The continuous subcutaneous infusion of basic fibroblast growth factor in rats with mechanically induced right ventricular hypertrophy, by promoting vascular angiogenes, modulates the response of the right ventricle, promoting the hypertrophy of contractile elements and limiting the areas in which fibrosis develops. Obviously, we cannot exclude that a small part of the fibrosis develops as scarring replacement of dead cardiomyocytes because of inadequate vascular supply or sustained stress.

The different effect produced by the basic fibroblast growth factor of the left and right ventricular myocardium in our rats is noteworthy. We believe that the vascular proliferative effect of basic fibroblast growth factor has been accentuated by the hypertrophic stimulus induced by the pulmonary artery banding on the right ventricle, which has not been present in the systemic left ventricle during the current experimental study.

Basic fibroblast growth factor, a mitogenic heparin-binding protein, has long been known to stimulate proliferation of cultured mesenchymal cells such as fibroblasts, endothelial cells, smooth muscle cells, and skeletal myoblasts, and is also involved in the regulation of cell survival, migration, and matrix production/degradation.[24–26]

Friehs et al demonstrated that by enhancing ventricular micro-vascular net with vascular endothelial growth factors, there is a normalising substrate delivery to myocytes, an improved tolerance to ischaemia, and a maintained glucose uptake rate.[27–29] Furthermore, Schultz et al reported that basic fibroblast growth factor knockout mice exhibited reduced interstitial fibrosis after aortic banding.[27] The fact that fibrosis and hypertrophy are important components of the heart's response to hypoxic or mechanical stress suggested that basic fibroblast growth factor might be an important regulator of myocardial repair.[28] Similar results have been reported by Shao et al;[30] however, the ability to maintain functional long-term improvement remains a challenge.[31]

This is the first study in which a basic fibroblast growth factor has been administrated continuously for the whole period of the study by subcutaneous implantation of osmotic pumps. It appears to be a valid and reproducible method for delivering systemic basic fibroblast growth factor to the heart tissues, which guarantees a constant stimulation of the right ventricular myocardium during the period of the study. Osmotic pumps were easy to implant and with no risk of complications when compared with other more invasive methods, such as intra-pericardial or direct intra-myocardial infusion.[21,32]

A limitation of this study is represented by the fact that basic fibroblast growth factor infusion gives a systemic vehiculation of the drug, which can possibly increase the risk of extracardiac "undesired effects". Small bowel segment, renal and hepatic tissue collection along with rat blood samples were obtained for further analysis.

Furthermore, on the basis of data from the literature, we have chosen to deliver basic fibroblast growth factor via osmotic pumps at a rate of 25 microlitres per hour for 28 days. Care should be taken to adjust the infusion rate for optimising the cardiac effect and avoid possible systemic affects.

In conclusion, the subcutaneous infusion with osmotic pumps is a valid and reproducible method of delivering basic fibroblast growth factor to the heart tissue in rats. The use of basic fibroblast growth factor leads to a higher capillary preservation in the right ventricular myocardium, which promoted the quality of compensatory right ventricular hypertrophy hampering the development of fibrosis.

Future studies are needed to understand the optimal basic fibroblast growth factor dosages to further ameliorate the right ventricular response to stress in failing hypertrophied right ventricles.

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