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Adenovirus-mediated overexpression of inhibitor kappa B-alpha attenuates postinfarct remodeling in the rat heart☆

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Abstract

Objective: The transcription factor nuclear factor kappa B (NF-kB) plays an important role in the inflammatory response following myocardial infarction. We hypothesized that NF-kB-blockade in an animal model of acute ischemia reduces the inflammatory response and therefore attenuates ventricular remodeling. **Methods**: Myocardial infarcts (MI) were produced in male Sprague–Dawley rats by ligation of the LAD and followed by adenovirus-mediated intramyocardial delivery of inhibitor kappa Bα-gene ($n=10$), the physiological inhibitor of the transcription factor nuclear factor kappa B, respectively, of a β-gal reporter-gene ($n=11$). Sham-operated animals ($n=10$) received neither ligation nor gene transfer. Five days after MI IkB-expression levels were determined by western blotting. Seven weeks after MI in vivo cardiac function was evaluated by transthoracic echocardiography. Based on left ventricular endsystolic and enddiastolic diameters ejection fraction and fractional shortening were calculated. Only animals with MI involving more than 30% of the left ventricle were included. Data are given as mean±SD. **Results**: In IkBα-transfected hearts IkBα-levels were six-fold higher ($P<0.05$) than in β-gal transfected hearts. Concerning in vivo hemodynamics IkBα-treated hearts showed reduced systolic and diastolic left ventricular dimensions compared to the β-gal MI-group (systolic 48±4 vs. 66±3 mm; diastolic 67±5 vs. 84±6 mm; $P<0.01$). Consequently fractional shortening (27.8±1.5 vs. 20.4±4.0%; $P<0.01$) and ejection fraction (63.4±3.6 vs. 49.1±8.3%; $P<0.05$) were preserved in IkBα hearts compared to β-gal MI-hearts. **Conclusion**: It can be concluded that overexpression of IkBα leads to an improved cardiac function thereby attenuating postinfarct remodeling.
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Keywords: Gene transfer; Myocardial infarction; Remodeling; NF-kappaB

1. Introduction

Since the transcription factor Nuclear Factor kappaB (NF-kB) had been discovered in 1986 there has been evolving evidence for its importance in the regulation of both physiological and pathological processes [1]. Among the factors regulated by NF-kB there are genes involved in immune and inflammatory responses, in the regulation of apoptosis, cell differentiation and cell growth [2]. Because of its pivotal role in inflammation modulation of NF-kB activity has turned out to be a promising target in treating diseases with uncontrolled inflammatory responses [3].

A common pathophysiological mechanism of major cardiovascular diseases such as atherosclerosis, myocardial infarction (MI), heart failure and myocarditis is a dysregulated inflammatory response. Different stress factors, including ischemia, oxidative stress, infection, cytoskeletal

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deformation, extracellular matrix alteration and cytokines, lead to the activation of different signaling pathways and downstream nuclear transcription factors, a prominent member being NF-kB. The activation of NF-kB is tightly regulated [4–6]. Normally NF-kB resides in an inactive state in the cytoplasm complexed to its inhibitor IkB. As response to stress factors different signaling pathways converge on the activation of IkB kinase complex (IKK), which induces phosphorylation of the IkB-molecule. This phosphorylation leads to the degradation of IkB by the proteasome and to its dissociation from its complex with NF-kB. Thereby, NF-kB is activated and enters the nucleus where it binds to the DNA at so-called kB binding sites.

Following coronary ligation, it could be shown that ischemia alone as well as ischemia and reperfusion cause the upregulation of inflammatory cytokines in both the infarcted area and the remote zone of the left ventricle, which is mainly orchestrated by NF-kB [7–11]. These cytokines and the consecutive inflammatory response, they are involved in, are considered to be essential in the early phase postMI for scar formation and tissue repair, but they exert detrimental effects on the structural and functional properties of the myocardium when they persist [12,13]. So far several attempts were made to inhibit NF-kB activation after the onset of ischemia by pharmacological and gene therapeutic means, resulting in a reduction of myocardial injury [14–17]. However, all these studies have in common a rather short observation period of some hours to a few days per maximum and concentrate on the effects of NF-kB inhibition in the early phase after the ischemic injury. In contrast, we were interested in the long-term effect of NF-kB inhibition on postinfarct remodeling. Therefore, an observation period of 7 weeks was chosen to make sure that all acute changes immediately following MI are finished, but the hearts are in the chronic phase of cardiac remodeling. It was the goal of the present study to test the hypothesis whether modulating NF-kB activity by overexpression of its inhibitor IkB exerts a long-term benefit on cardiac function even in the chronic state of remodeling.

2. Materials and methods

2.1. MI protocol

MI was induced in adult male Sprague–Dawley rats (395 ± 26 g) by ligating the left anterior descending coronary artery according to the technique of Pfeffer et al. [18]. The animals were anesthetized intraperitoneally with a mixture of xylazin (1 mg/100 g bodyweight) and ketamin (10 mg/100 g bodyweight), intubated with a fine polyethylene tube and ventilated mechanically with a rodent ventilator using oxygen and room air (60 min^{-1}). When the chest was opened 0.2% isofluran was added to the oxygen–air mixture.

The chest was entered via a left lateral thoracotomy in the fourth intercostal space, the beating heart was visualized and a ligature was placed around the LAD beneath the left auricle. Immediately after the onset of ischemia gene transfer was performed according to the protocol described in Section 2.2. Then the chest was closed after being evacuated by a small polyethylene cannula. Sham-operated rats underwent the same procedure, except no suture was placed around the LAD and no gene transfer was performed. The perioperative mortality in the first 48 h was about 30%. All animals had free access to standard rat chow and water during the observation period of 7 weeks and received humane care in compliance with the European Convention on Animal Care. The experiments were approved by the committee for animal research, Medical University of Vienna.

2.2. Adenovirus constructs

For overexpression of IkBa we used an adenoviral construct containing the coding sequence for porcine IkBa under the control of the cytomegalovirus promoter (rAd.IkBa). Briefly, the IkBa coding region was amplified by PCR with a nuclear localization sequence (NLS) fused to its 5′ end. The construct was cloned into the adenovirus transfer vector pACCMVpLpASR(+) as described [19], and recombination with pJM17 was carried out in HEK293 cells to yield a recombinant replication-deficient type 5 adenovirus with the IkBa construct inserted into the E1 region. The construction is described in detail in Wrighton et al. [20]. As control adenoviral construct a recombinant adenovirus containing the coding sequence of β-galactosidase (rAd.β-gal) under the control of the Rous Sarcoma Virus promoter was used which is described in Stratford-Perricaudet [21].

2.3. Intramyocardial gene delivery

The animals with MI were randomised and allocated to two different groups. In the treatment group MI was followed by the transfer of the rAd.IkB-vector ($n = 10$), the control group was transfected with a β-gal reporter-gene ($n = 11$) as described by Bernecker et al. [22]. For direct gene delivery, the heart was exposed and the pale area of the left ventricular free wall was identified as the infarcted myocardium. A total of 1.56×10^9 pfu of rAd.IkB and rAd.β-gal, respectively, in a final volume of 250 μl were delivered by a 25-gauge needle into five sites around the ischemic area directly into the myocardium of the periinfarct zone. To avoid puncture of the ventricle an injection depth of about 1.5 mm was chosen. This depth was guaranteed by using a special, custom-designed device with a flexible plate connected to the needle (Fig. 1). After injection the heart was monitored for 2–3 min for resumption of normal sinus rhythm.

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Fig. 1. Application device.

2.4. In vivo hemodynamic measurements

At baseline before the induction of MI and 7 weeks postMI, in vivo hemodynamics were assessed by transthoracic echocardiography. Echocardiographic studies were carried out under light anesthesia and spontaneous respiration with xylazin (1 mg/100 g bodyweight) and ketamin (10 mg/100 g bodyweight) using the same dose in both MI and sham-operated rats. The chest was shaved, the animals were placed on their left side and copious quantities of transducer gel were applied to the animals' fur to optimize ultrasound transmission. Imaging was performed by an ultrasonographer experienced in rodent imaging with a 7.5 MHz commercially available standard pediatric transducer connected to a echocardiographic computer console (VINGMEDSound, CFM 800, software version 1.0). Phased array technology with a spatial resolution of 0.2 mm was used. The interrogation depth was set at 4 cm.

A parasternal long axis view was followed by a parasternal short axis view. After a good image quality of the mid-papillary muscle level of the left ventricle in two-dimensional echocardiography had been obtained M-mode was added for measurements of left ventricular dimensions at end-systole and end-diastole. Fractional shortening and ejection fraction were calculated. The measurements of three heart beats were performed online from the screen and averages were used for further analysis.

2.5. Infarct size and tissue collection

Immediately after the collection of in vivo hemodynamic data the animals were heparinized intravenously (200 IU) and the hearts were excised quickly and put into ice-cold saline. Total heart weight was determined, the atria were separated from the ventricles and weighed, left ventricular (LV) and right ventricular (RV) weight were determined separately, the infarcted area of the LV was dissected from the non-infarcted area and each was weighed. Infarct size was expressed as the ratio of infarct to total LV mass. Only animals with MI bigger than 30% of the left ventricle were included in our study. Tissue samples were snap frozen in liquid nitrogen and stored at −80 °C.

2.6. Western blot analysis

Five days postMI, five animals per group were killed to determine IkBα-expression levels by SDS-PAGE and western blotting. The myocardial tissue samples were minced at 4 °C in phosphate buffered saline (PBS) where a protease inhibitor at a concentration of 20 mmol/l was added (Pefabloc SC Pentapharm Ltd) homogenized and centrifuged at 13,000 rpm for 10 min. The supernatant was centrifuged at 13,500 rpm for 20 min, and the final supernatant was used for further analysis. The protein concentration was determined by Coomassie brilliant blue staining (Coomassie Brilliant Blue Reagent G-250 based, Pierce, USA). The samples were separated in SDS-polyacrylamide gradient gels with a concentration from 3.6 up to 15%. After separation proteins were electro-blotted onto nitrocellulose membranes (Protran western blotting membrane, Schleicher and Schuell) and blocked with 20% fetal calf serum (FCS) in TRIS-buffered saline (50 mmol/l TRIS, pH 7.4, 150 mmol/l NaCl) with 0.02% Tween 20 (TBST) for 1 h at room temperature. Afterwards the membranes were incubated with the primary IkBα antibody (IkB-α Antibody, Cell Signaling Technology) at a dilution of 1:500 in 20% FCS in TBST overnight at 4 °C. The membranes were washed in TBST for 30 min with four changes of buffer and incubated with a specific horseradish–peroxidase coupled secondary antibody (HRP-conjugated goat anti-rabbit IgG, JAXELL) diluted 1:4000 in 20% FCS in TBST for 1 h at room temperature. The membranes were again washed for 30 min in TBST with four changes of TBST. Signals were detected using enhanced chemoluminescence (ECL plus Western Blotting Detection Reagents, Amersham Pharmacia Biotech) and finally quantified using a densitometer with imaging system (LumiAnalyzer, Boehringer Mannheim). The membranes were treated with 1.54 g dithiothreitol, 10 ml 20% SDS, 5 ml 1 M Tris–HCl, pH 6.8 for 30 min, washed with TBST for 30 min with four changes of buffer and blocked with 20% FCS in TBST for 1 h at room temperature. Then the membranes were incubated using primary antibodies against actin (Anti-Actin Rabbit Polyclonal Antibody, Trevigen) 1:1000 diluted in 20% FCS in TBST at 4 °C overnight. Densitometric signals were determined according to the protocol described above. IkB signals were normalized to the signals of actin to eliminate potential differences in the protein content of each lane. Results are presented as IkB to actin signal ratio. Prestained molecular weight standards (Prestained SDS-PAGE Standards, BioRad) were used to ensure adequate protein separation and transfer.

2.7. Statistical analysis

All data are expressed as mean ± standard deviation (SD). To compare differences between groups one-way ANOVA coupled with Bonferroni multiple comparison test was used. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using the SPSS statistical software package (version 10.0 for windows).

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3. Results

3.1. Survival postMI and gene transfer

In the two groups which received MI followed by the transfer of the IkBα gene and a β-gal reporter gene, respectively, postoperative mortality was about 30% in the first 48 h while no difference in mortality between treatment and control MI group could be observed. All sham-operated animals survived the operation. In total, 31 animals survived the first 48 h. Rats with MI less than 30% of the left ventricle were excluded from further analysis, these were four animals in the IkB-treated group and three animals in the β-gal transfected group. At random 15 animals were sacrificed after 5 days to determine transfection efficiency indicated by IkB expression levels and 16 rats completed the study protocol of 7 weeks.

3.2. IkBα expression

Five days postMI and gene transfer or the respective procedure in sham-operated animals IkBα expression was analysed by western blotting. In all the samples taken from the periinfarct zone or the respective area in sham-operated animals of the LV free wall IkBα expression could be detected (Fig. 2). The primary IkBα antibody used recognizes both endogenous and via gene transfer expressed IkB. Compared to the sham-operated animals ($n=5$) both infarct groups showed significantly reduced IkB levels ($P<0.01$), nevertheless in the IkB treated infarct group ($n=5$) a six-fold higher IkB expression could be achieved by direct intramyocardial gene delivery than in the β-gal transfected infarct group ($n=5$, $P<0.05$) (Fig. 3). To eliminate potential differences in the protein content loaded densitometric signals of IkB were normalized to the densitometric signals of actin. Results are presented as IkB to actin signal ratio.

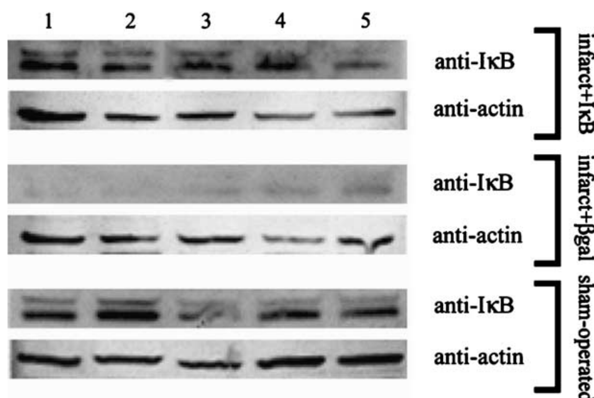

Fig. 2. Representative immunoblots for IkB 5 days postMI in the periinfarct zone and the corresponding area of the LV free wall in sham-operated animals, respectively. From top to bottom: infarct+IkB, infarct+β-gal, sham-operated. Densitometric signals of IkB were normalized to those of actin. Summary data are presented in Fig. 3.

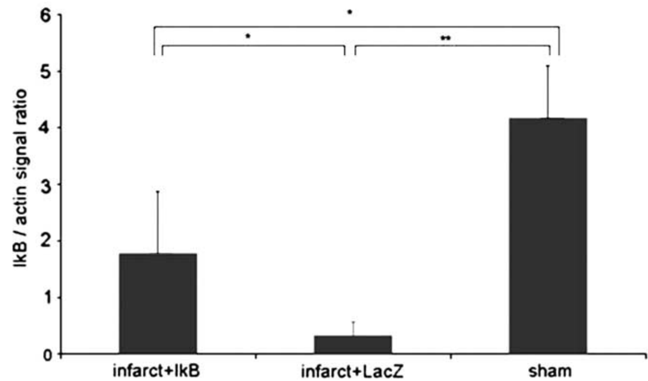

Fig. 3. IkB expression levels 5 days postMI in the periinfarct zone and the corresponding area of LV in sham-operated animals, respectively. Data are presented as IkB to actin signal ratio. $N=5$ per group, $^{*}P<0.05$, $^{**}P<0.01$.

3.3. Morphometrics

Infarct size was comparable between IkB treated and β-gal transfected animals with 45.9±8.9 vs. 45.4±8.5% of total LV mass (Table 1). Bodyweight and tibia length were similar in all three study-groups. Seven weeks postMI in the infarcted animals, no difference in LV to bodyweight ratio (3.4±0.7 vs. 3.1±0.3; $P=$non-significant (NS)) and RV to bodyweight ratio (0.8±0.2 vs. 0.9±0.2; $P=$NS) could be observed between IkB treated and β-gal transfected group. Compared to sham-operated animals, however, both infarct groups showed significantly elevated LV to bodyweight ratio and a tendency towards a higher RV to bodyweight ratio.

3.4. In vivo hemodynamics

Seven weeks postMI and gene transfer, both infarct groups showed significantly higher LV diameters in systole and diastole compared to the sham-operated animals assessed by transthoracic echocardiography. However, LV dilatation was less pronounced in the IkB treated infarct group compared to the β-gal transfected animals, observed both for diastolic (67±5 vs. 84±6 mm; $P<0.01$) and for systolic values (48±4 vs. 66±3 mm; $P<0.01$) (Figs. 4 and 5). Based on these dimensions fractional shortening and ejection fraction were calculated as parameters of cardiac function. In the infarcted animals, fractional shortening as well as ejection fraction were significantly enhanced in the treatment group compared to the control group (27.8±1.5 vs. 20.4±4.0%; $P<0.01$ and 63.4±3.6 vs. 49.1±8.3%, respectively, $P<0.05$) (Fig. 6) as a consequence of preservation of ventricular geometry.

4. Discussion

The present findings show that a reduction of NF-kB activity by overexpression of its inhibitor IkB postMI

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Table 1
Morphometric measurements 7 weeks postMI

	MI		
	Sham-operated ($n=5$)	Infarct + IkB ($n=6$)	Infarct + β-gal ($n=5$)
Infarct size, % total LV weight	n.a.	45.9 ± 8.9	45.4 ± 8.5
Body weight (g)	455 ± 55	471 ± 41	457 ± 22
Tibia length (mm)	46.8 ± 1.3	47.3 ± 1.0	46.9 ± 0.7
Total heart weight (g)	1.89 ± 0.23	2.43 ± 0.31*	2.33 ± 0.22*
LV weight (g)	1.13 ± 0.10	1.58 ± 0.17*	1.44 ± 0.13*
LV-to-bodyweight ratio	2.5 ± 0.2	3.4 ± 0.7*	3.1 ± 0.3*
RV weight (g)	0.33 ± 0.5	0.39 ± 0.08	0.39 ± 0.7
RV-to-bodyweight ratio	7.3 ± 2.2	8.3 ± 2.2	8.7 ± 1.8

Values are means ± SD. *$P < 0.05$ vs. sham.

attenuates postinfarct remodeling and improves cardiac function, thus providing proof of the concept that (1) localized gene expression meets the needs of a localized pathology such as MI and is sufficient to improve global ventricular function and that (2) the effect of IkB expression with reducing the inflammatory response postMI outlasts the entire expression period of the adenovirus used in this study [23,24].

Five days postMI and gene transfer, both infarct groups showed significantly reduced IkB levels, compared to the sham-operated animals, where no IkB degrading stress stimuli occurred. However, IkB expression in the infarct group treated by IkB gene transfer was six-fold higher than in the β-gal transfected control group. We chose direct intramyocardial instead of intracoronary gene delivery because (1) of its superior transfection efficiency in the limited area surrounding the infarct zone, where the major part of the inflammatory response postMI takes place and (2) because of its clinical relevance for cardiac surgery. It has been shown by our group and others that gene transfer

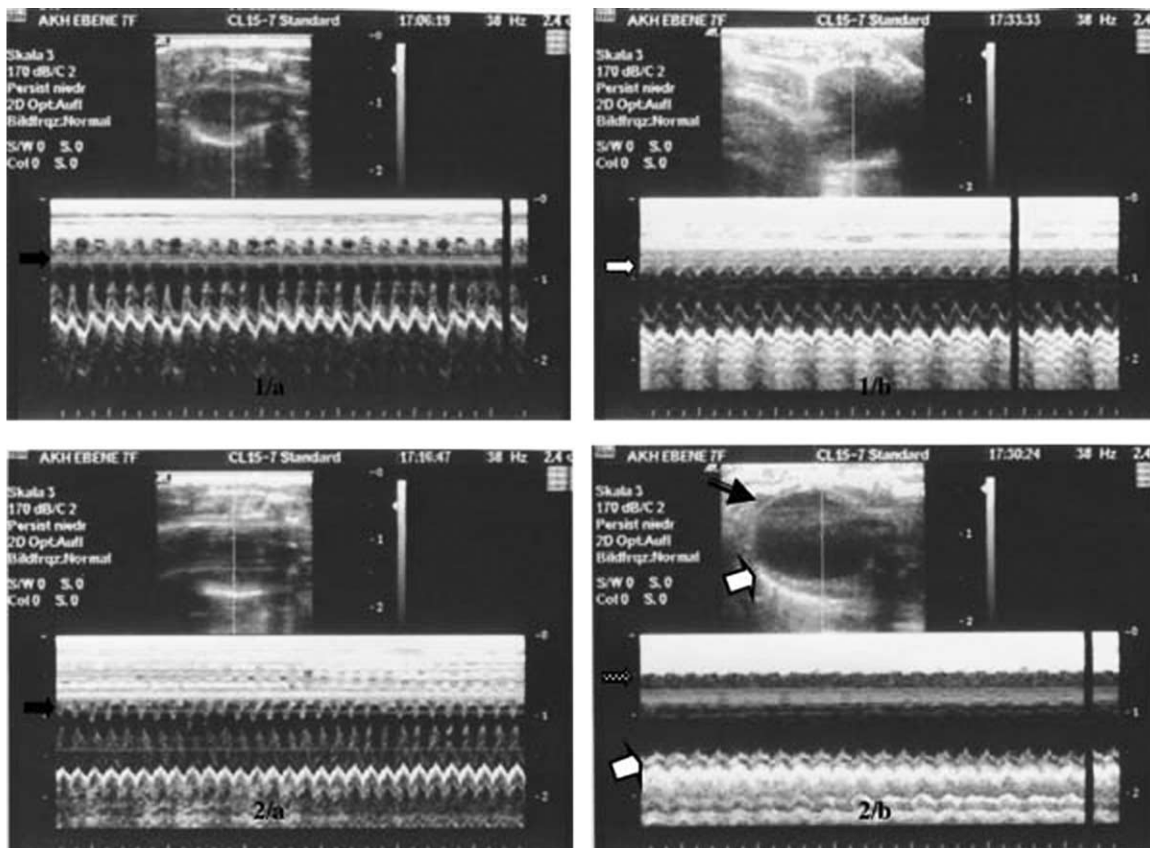

Fig. 4. M-mode echocardiography of a rat heart at baseline (1a, 2a) and 7 weeks after ligation of the left anterior descending coronary artery, after treatment with IkB (1b), and without treatment (2b). Black arrows show normal septal motions at baseline (1a and 2a), white arrow indicate hypokinesia of the septum, dotted arrow shows akinesia (2b). The heart dilated, the septal and anterior wall are thin and akinetic (upper large black arrow in 1b), and the posterior wall is hypokinetic (large white arrow), as the consequence of the left ventricular dilatation and remodeling.

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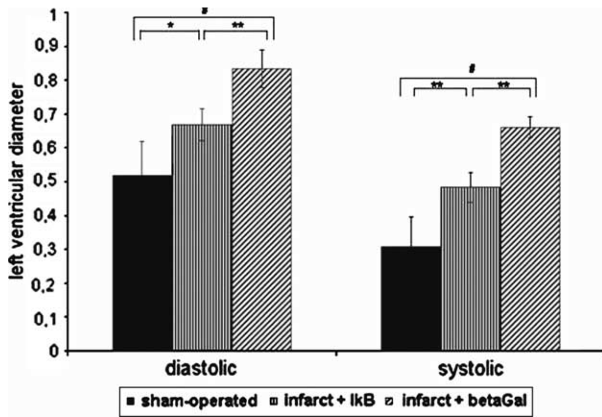

Fig. 5. Effect of IkB treatment on left ventricular (LV) chamber dilatation 7 weeks postMI. LV enddiastolic (left) and endsystolic (right) diameters were determined by transthoracic echocardiography. $N=5$ in infarct+IkB, sham; $n=6$ in infarct+ β-gal; $^{**}P<0.01$, $^{\#}P<0.001$.

by direct injection into the myocardium leads to a local gene expression in the vicinity of the injection site with >80% of the myocytes expressing the transfected gene [25,26]. Due to current literature adenoviral vectors have a characteristic expression profile with peak expression levels between days 3 and 6 after transfection and no detectable transgene expression after about 4 weeks. Therefore, in our study analysis of IkB expression levels 5 days after MI and gene transfer coincides with the peak expression of the adenoviral vector used whereas 7 weeks postMI and transfer no further transgene expression can be expected. The dose of 1.56×10^9 pfu of the LacZ and IkB vector, respectively, was found in a preliminary study by our group, prove to be effective and confirmed the adenoviral expression profile described above both in infarcted and non-infarcted myocardium (data not shown).

Seven weeks postMI the IkB treated hearts showed significantly reduced LV dimensions, both for systolic and diastolic values compared to the β-gal transfected control hearts. Consequently fractional shortening and ejection

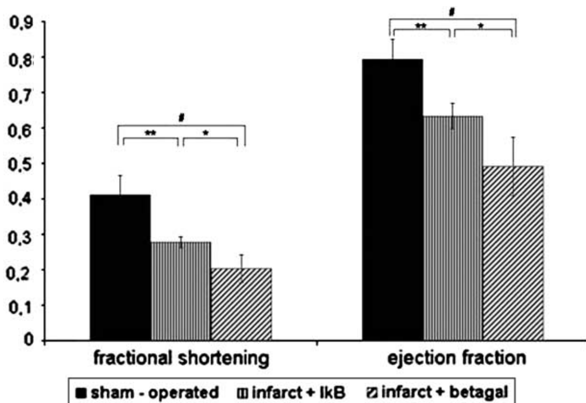

Fig. 6. Effect of IkB treatment on cardiac function 7 weeks postMI. Fractional shortening (left) and ejection fraction (right) were calculated based on LV dimensions. $N=5$ in infarct+IkB, sham; $n=6$ in infarct+ β-gal; $^{*}P<0.05$, $^{**}P<0.01$, $^{\#}P<0.001$.

fraction as parameters of cardiac function were significantly higher in the treatment group compared to the β-gal transfected control group. Using proteasome inhibitors to block NF-kB activation and oligonucleotid decoys to scavange activated NF-kB, respectively, in pig and rat models of MI a significant reduction of infarct size could be achieved in the treated groups compared to the non-treated groups [14–17]. Evaluation of cardiac function revealed positive effects on LV functional properties. In a pig model with 1 h of ischemia followed by 3 h of reperfusion preservation of regional myocardial function measured by segmental shortening was observed [13]. In the isolated rat heart the administration of a proteasome inhibitor in the setting of 20 min of ischemia followed by 45 min of reperfusion lead to an improvement in LV developed pressure and contractility [16]. A downregulation in the transcription of the kB-dependent cell adhesion molecules ICAM-1, VCAM-1, ELAM and P-Selectin and of the proinflammatory cytokines IL-6 and IL-8, which results in a reduced accumulation of leukocytes, was considered to be the molecular basis of functional improvement [13,15,16].

All these studies investigated the effects of NF-kB inhibition in the setting of acute ischemic injury over a few hours. In contrast, we were interested in the effect of NF-kB inhibition on the development of chronic heart failure due to postinfarct remodeling. On the one hand we could show that IkB overexpression by gene transfer postMI reduces LV dilatation and thereby improves cardiac function over a long-term observation period. On the other hand, however, we did not see any effect on cardiac hypertrophy evaluated by LV and RV weight to bodyweight ratio. So one may speculate that an initial attenuation of the inflammatory response helps to overcome the vicious circle started and maintained by the factors involved in the inflammatory cascade with its detrimental effects on the functional and structural integrity of the extracellular matrix of LV myocardium.

In contrast to the studies mentioned above focussing on the acute effects of NF-kB blockade on the development of the infarcted area we did not see any difference in infarct size because in our study a change in infarct size could not be determined due to methodological characteristics. We chose ligating the LAD as infarct model because it induces heart failure if more than 30% of the left ventricle is involved. It was important to us to ensure that MIs are comparable between groups and hemodynamically relevant in order to cause ventricular remodeling. So this cut-off point of 30% was chosen being aware of the fact that we probably eliminate an additional positive effect of reducing NF-kB activity on infarct size. Once more we were not interested in acute effects, but in the effects of IkB treatment on the development of ventricular remodeling.

The particular mechanisms behind the morphological and physiological benefits in the treatment group described in this study can so far only be postulated according to the literature cited above. The influence of overexpressing IkB

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on gene programs orchestrated by NF-kB needs further investigation and has to be proven on the molecular level. These issues shall be addressed in future studies and will probably provide further insight in the complex mechanism of postinfarct remodeling.

Compared with other gene therapeutic approaches for the treatment of heart failure this novel strategy offers the benefit that a single intervention during the early stage of MI leads to a positive effect on the development of LV remodeling and cardiac function, which can be maintained even in the state of chronic heart failure. Currently the treatment of heart failure by the means of gene therapy is confronted with the problem that a long lasting expression at high expression level is needed to develop biologically relevant effects for the transfer of genes encoding for structural proteins. These prerequisites can hardly be achieved by currently available vectors. The combination of the adenoviral vector with a delayed onset of activity and the blockade of a central factor of the inflammatory cascade seems to have both transient gene expression and effective anti-inflammatory potential. This therapeutic concept could serve as the basis for a potential preventive strategy for patients with large MI and the future risk of developing heart failure.

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Appendix A. Conference discussion

Dr J. Vaage (*Oslo, Norway*): I'd like to ask you one thing. There are also pharmacological agents that will inhibit NF-kB. Have you tested any of those?

Dr Trescher: No. We just tested by gene transfer.

Dr R. Poston (*Baltimore, MD, USA*): IkB acts intracellularly, so does it work if you give it exogenously, or only in those cells that take up the gene and express it intracellularly?

Dr Trescher: Could you repeat your question.

Dr Poston: Only the cells that get transfected with the gene, do they get the protective effect of IkB, or do the adjacent cells that don't take up the gene also get protected?

Dr Trescher: IkB is expressed in each cell. And by this gene transfer, we overexpress IkB. So it is overexpressed in every cell.

Dr Poston: Well, you showed us the transfection efficacy with the high levels of IkB, but you didn't show us efficiency. In other words, how many cells actually took up the gene? I noticed you used the LacZ reporter gene, so you have the data. I suspect the efficiency was fairly poor with direct needle injection of the myocardium. Poor efficiency of delivery has been one of the main problems with gene therapy in general.

Dr Trescher: Yes. But we inject directly into the myocardium and so we have a very high transfection efficiency around these needle tracks.

Dr Poston: I'm sorry to keep belaboring this point, but what percentage of cells in the infarct zone took up the gene?

Dr Trescher: We didn't inject it into the infarct zone because there are probably no viable cells and we can't expect any IkB expression. So it's injected into the viable myocardium.

Dr J. Vaage: Do you believe that the results would have been different if you actually had injected the gene into the heart before you did the infarct? Now, you did it at the same time.

Dr Trescher: It depends on the period. You inject it prior to the infarction. I think a very important point of this study is that we have a period of about 48 h postMI in which the gene isn't expressed due to the expression profile of the adenovirus. So I think this is the difference to pharmacological interventions. I think this time period is very important.

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