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Retroinfusion of NF κ B decoy oligonucleotide extends cardioprotection achieved by CD18 inhibition in a preclinical study of myocardial ischemia and retroinfusion in pigs

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Myocardial reperfusion injury is partially mediated by postischemic inflammation. Beyond acute PMN recruitment, postischemic inflammation comprises subacute PMN adhesion, eg via NF κ B activation. In a pig model of 60-min LAD occlusion by PTCA balloon inflation and 1 to 7 days of reperfusion, we investigated the impact of targeted NF κ B decoy oligonucleotide (ODN) transfection in the area at risk (AAR) on infarct size and regional myocardial function. After 55 min of LAD occlusion, liposomes containing NF κ B ODN were selectively retroinfused into the anterior interventricular vein for 5 min. Then, retroinfusion was stopped and reperfusion was initiated. Where indicated, CD18 antibody IB4 was infused systemically at 30 min of ischemia. Methylene blue and tetrazolium-red staining were used for quantification of the infarct size. Subendocardial segment shortening (SES)

by sonomicrometric crystals in infarct area and AAR was assessed under pacing (expressed as % of control region). NF κ B decoy ODN retroinfusion reduced infarct size ($36 \pm 4\%$ versus $49 \pm 5\%$ in control hearts at day 7), whereas functional reserve of the AAR (SES $73 \pm 17\%$ versus $46 \pm 18\%$ at 180/min) tended to improve. Similar effects were observed after IB4 infusion ($38 \pm 5\%$ infarct size, $85 \pm 7\%$ SES at 180/min). A combination of NF κ B decoy ODN retroinfusion and IB4 infusion further decreased infarct size ($26 \pm 2\%$) and improved functional reserve (SES $94 \pm 6\%$ at 180/min). We conclude that NF κ B decoy ODN transfection by retroinfusion is feasible in pig hearts and provides postischemic cardioprotection in addition to CD18 blockade. Gene Therapy (2002) 9, 518–526. DOI: 10.1038/sj/gt/3301673

Introduction

Reperfusion of an occluded coronary artery, established as standard treatment in the case of myocardial infarction, may induce arrhythmias, myocardial stunning and microcirculatory flow disturbances, such as no reflow in the postischemic myocardium. Each of these clinically relevant phenomena has been associated with release of reactive oxygen species from recruited polymorphonuclear neutrophils (PMN).^{1–3}

PMN adhesion may occur rapidly after onset of ischemia. Accordingly, early bolus application of adhesion antagonists, eg CD18 antibodies or selectin antagonists, reduces myocardial detriment after 3–6 h of reperfusion.^{4,5} However, improvement of myocardial function after anti-adhesive therapy fades over time, and is maintained only in animals receiving continuous anti-adhesive treatment.^{6,7}

Consistent with these observations, postischemic PMN adhesion occurs in an immediate or delayed manner:

rapid post-translational modification of for example, P-selectin or PAF provides acute adhesion, whereas prolonged leukocyte recruitment is achieved by transcription of proinflammatory proteins in the postischemic tissue. For example, subacute endothelial activation provides adhesion molecules, cytokines and chemokines. Characteristically, induction of these proteins is regulated by NF κ B,⁸ a factor sensitive to ischemia and reperfusion *ex vivo*⁹ and *in vivo*.¹⁰ The inhibition of the family of NF κ B-dependent proinflammatory genes has been found to exert a more substantial cardioprotection after ischemia and reperfusion than antisense ODN directed *versus* a single proinflammatory gene.¹¹ Selective NF κ B inhibition may be provided by decoy transfection, loading targeted cells with short, double-stranded oligonucleotides which encode the DNA-binding sequence of NF κ B.¹² The exogenous decoy oligonucleotide competes nuclear promoter binding sites for the activated NF κ B protein, inhibiting its transcriptional potential in cultured cells,¹³ *ex vivo*⁹ and *in vivo*.¹¹ Of note, NF κ B decoy blockade, when administered concomitantly with the stimulus, does not inhibit acute leukocyte adhesion.¹⁴ In contrast, NF κ B decoy ODN transfection is capable of blocking subacute PMN adhesion to the postcapillary venules of isolated hearts 8 h after myocardial ischemia.⁹

An additive effect of inhibiting acute, as well as sub-acute endothelial activation might critically extend the therapeutic strategies for myocardial protection against reperfusion injury, whereas patient outcome has not improved so far when solely acute endothelial activation was inhibited.^{15,16} To test the hypothesis that subacute endothelial activation is a significant therapeutic target in the context of postischemic myocardial reperfusion injury, efficient regional transfection of decoy oligonucleotides into ischemic myocardium is essential. The latter is of crucial importance, because the size of the human myocardium exceeds that of a rat about 250-fold, rendering intracoronary arterial injection of decoy ODN unfeasible. Recently, we demonstrated that myocardial reporter gene expression was 50-fold enhanced after retroinfusion using the coronary vein for delivery compared with intracoronary arterial injection.¹⁷ In this study, selective pressure-regulated retroinfusion into a coronary vein (AIV) was performed during occlusion of the corresponding coronary artery (LAD), mimicking the clinical setting of coronary occlusion and recanalization. Using this catheter-based approach, we now infused liposomes carrying NFκB ODN into the ischemic myocardium with or without systemic infusion of a CD18 antibody.

Results

NFκB decoy oligonucleotide transfection

In a cell culture model using transfection of a construct containing luciferase, NFκB decoy transfection in endothelial cell culture substantially reduced TNFα or PMN-dependent NFκB activation, when applied simultaneously with these stimuli. When NFκB decoy ODN transfection was performed 24 h before stimulation, the inhibitory potential was reduced by 44%. A nonsense oligonucleotide (scrambled ODN) had no effect on TNFα-dependent NFκB activation.

In vivo, we aimed at efficient NFκB decoy oligonucleotide (ODN) delivery to the ischemic myocardium in a pig model. For this purpose, we infused NFκB decoy ODN containing liposomes retrogradely into the vein draining the ischemic area, adjusting the retroinfusion pressure to the individual coronary venous anatomy.¹⁷ With this approach, a significant portion of endothelial cells in the targeted area at risk were transfected with ODN 24 h after ischemia, whereas no transfection occurred in the control area (Figure 1b). After 7 days of reperfusion, no ODN was detectable in the area at risk.

When NFκB decoy ODNs were retroinfused at the end of ischemia, NFκB activation was blocked 1 h after reperfusion in the infarcted area, as well as in the area at risk. After 24 h of reperfusion, the increase in NFκB activation was attenuated, resembling the endothelial cell culture kinetic (Figure 1d). After 7 days, NFκB-binding activity in untreated and NFκB-treated postischemic myocardium decreased, without displaying a difference between experimental groups (Figure 1d). Compared with non-ischemic myocardium of the same heart, the infarcted area displayed an increase in TNFα and E-selectin mRNA 24 h after reperfusion (Figure 2). The increase was blunted by NFκB decoy. These findings indicate that the NFκB decoy strategy inhibited induction of NFκB-regulated genes in the targeted myocardium after ischemia and reperfusion.

Leukocyte recruitment in the reperfused myocardium

The anti-inflammatory effect of NFκB inhibition was assessed by quantification of the PMN influx into the reperfused myocardium. As demonstrated in Figure 3, leukocyte recruitment was substantially increased in the infarct area at 24 h of reperfusion, and remained above control level after 7 days. The leukocyte influx into the infarcted area was significantly reduced by NFκB decoy ODN treatment. Addition of IB4 did not enhance the inhibitory effect of NFκB decoy significantly.

Apoptosis induction in the reperfused myocardium

NFκB inhibition may induce apoptosis in the myocyte cell compartment treated with an NFκB inhibiting agent.¹⁸ Therefore, we analyzed if NFκB decoy ODN transfection increased the occurrence of apoptotic cells in the postischemic myocardium. As depicted in Figure 4, no excess apoptosis was detectable in NFκB decoy ODN + IB4-treated hearts. In contrast at 24 h, untreated hearts displayed more apoptotic cell nuclei than hearts treated with NFκB decoy ODN + IB4. At 7 days of reperfusion, no significant difference between both groups was detected.

Infarct size

As depicted in Figure 5a, 60 min of LAD occlusion resulted in an infarct area of $58 \pm 5\%$ of the area at risk after 24 h of reperfusion. Application of NFκB decoy ODN alone reduced infarct size to $38 \pm 5\%$, and did not decline significantly further, if IB4 was administered in addition to NFκB decoy ODN ($33 \pm 3\%$).

At day 7 of reperfusion, infarct size of control hearts was $49 \pm 5\%$, declining to $36 \pm 3\%$ after NFκB retroinfusion alone (Figure 5c). Systemic IB4 infusion without further treatment reduced infarct size to about the same extent as NFκB ODN alone ($37 \pm 4\%$), whereas an additive effect was found in the NFκB ODN + IB4-treated group ($26 \pm 2\%$). In contrast, retroinfusion of a scrambled ODN, did not influence infarct size achieved by IB4.

Global myocardial function

Hemodynamic measurements revealed a similar baseline values in all groups (Table 1). After 60 min of ischemia and 7 days of reperfusion, the control group showed a significantly decreased dP/dt_{max} and dP/dt_{min} compared with preischemic values. Treatment with either NFκB ODN retroinfusion, IB4 infusion or both induced a trend towards less deterioration without statistical significance (Table 1).

Regional myocardial function

To assess myocardial function in the region exposed to ischemia and reperfusion, sonomicrometry was performed in a standardized manner in the infarcted and non-infarcted area at risk, as well as in the nonischemic CX perfusion area. As depicted in Figure 6a, subendocardial segment shortening of the infarcted area was less than 50% of the nonischemic area in all groups under resting conditions and further declined with increased pacing rates, without significant differences between experimental groups.

In the area at risk, regional myocardial function was well preserved at rest (Figure 6b). As expected, pacing revealed a decreased functional reserve ($47 \pm 15\%$ at 180/min) in hearts subjected to ischemia and reperfusion

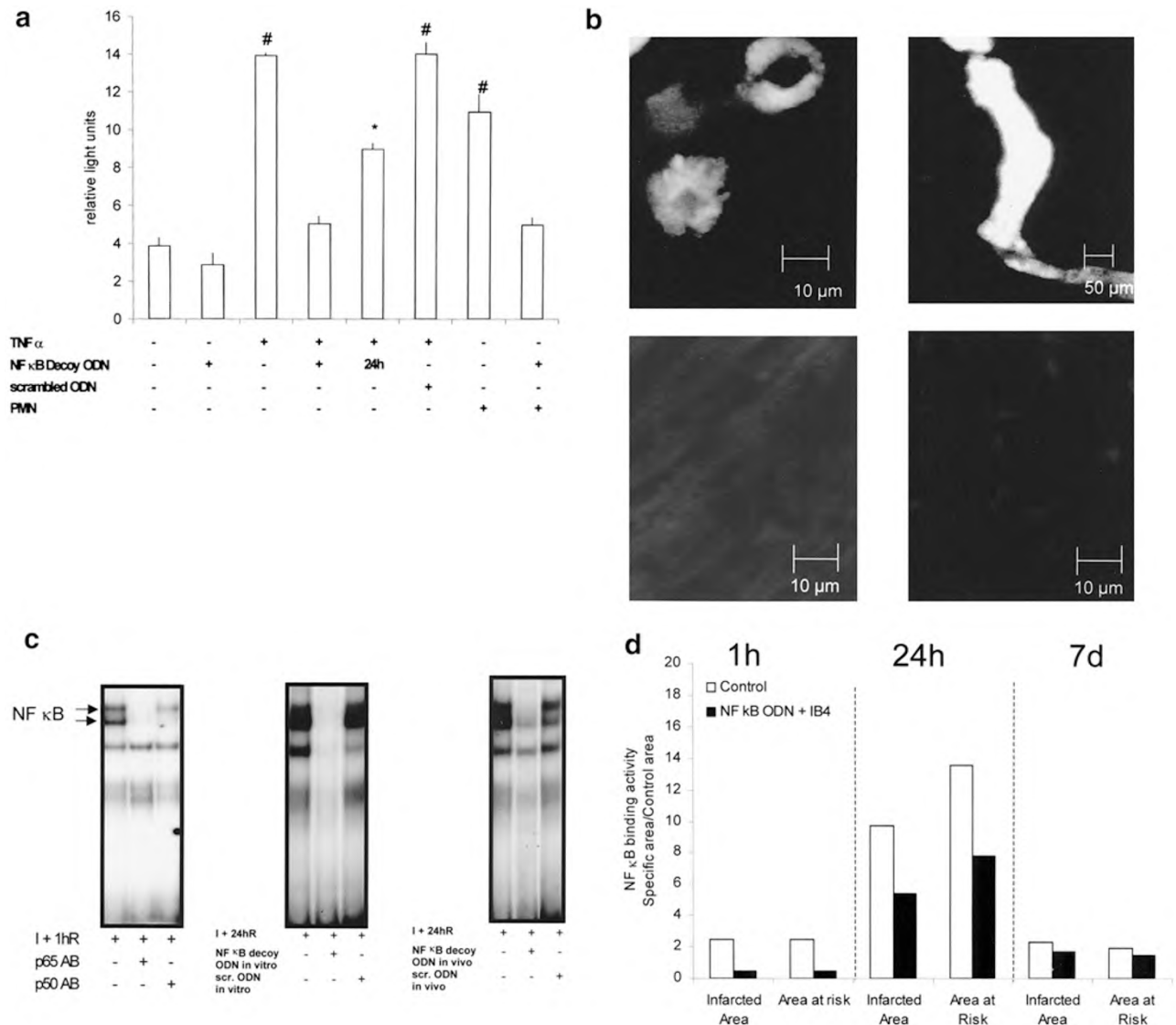


Figure 1 NFκB decoy ODN transfection specifically inhibits NFκB activation. (a) Rat coronary endothelial cells were transfected with a truncated, NFκB-sensitive ICAM-1-promoter-luciferase-cDNA construct and a receptor tyrosine kinase-renilla-luciferase construct. 24 h later, cells were stimulated with TNFα or PMN (500 000/well) for 24 h and then analyzed for luciferase activity (% of renilla activity). NFκB decoy ODN was transfected either simultaneously with stimulation (+) or 24 h before stimulation (24 h). Results are given from three independent experiments. *P < 0.05 versus unstimulated controls. (b) 24 h after retroinfusion, fluorescence microscopy of AMCA-stained NFκB decoy ODNs (left panel) revealed transfection of small vessels (12–20 μm diameter) in the area at risk, whereas rhodamine-stained ODNs were found in the wall of large vessels (50–100 μm) in the same area. Myocardium of the control region (Cx perfusion area) myocyte tissue did not contain rhodamine-stained ODNs. Two different dyes were necessary due to autofluorescence. (c) Electromobility shift assays (EMSA) reveal that NFκB protein isolated from infarcted pig myocardium consists of p65 homodimers (left panel, upper band, deleted by a p65 antibody) and a p50/p65 heterodimer (left panel, lower band). Specific inhibition of NFκB DNA binding is achieved ex vivo with an excess of unlabeled NFκB decoy ODNs, but not with scrambled ODNs. Right panel: in vivo, NFκB decoy ODNs, but not scrambled ODNs reduce DNA-binding. (d) NFκB DNA binding of tissue from the infarcted area and the area at risk, normalized to control region. (Representative examples of three independent experiments at each time-point).

without treatment. Functional reserve of the area at risk was significantly improved solely when NFκB ODN and IB4 administration were combined.

Discussion

In the present study, we used a pig model of 60 min myocardial ischemia with subsequent reperfusion up to 7 days to investigate the effect of acute NFκB inhibition on

infarct size and myocardial dysfunction. We took advantage of a novel percutaneous transluminal retrograde gene delivery system to target NFκB decoy oligonucleotides to the ischemic myocardium.¹⁷ Whereas untreated control hearts displayed a substantial increase in NFκB-binding activation and leukocyte recruitment after 1 day of reperfusion, NFκB inhibition by transfection of a suitable decoy ODN (see Figure 1c and d) reduced transcription of proinflammatory proteins (Figure 2), as well as

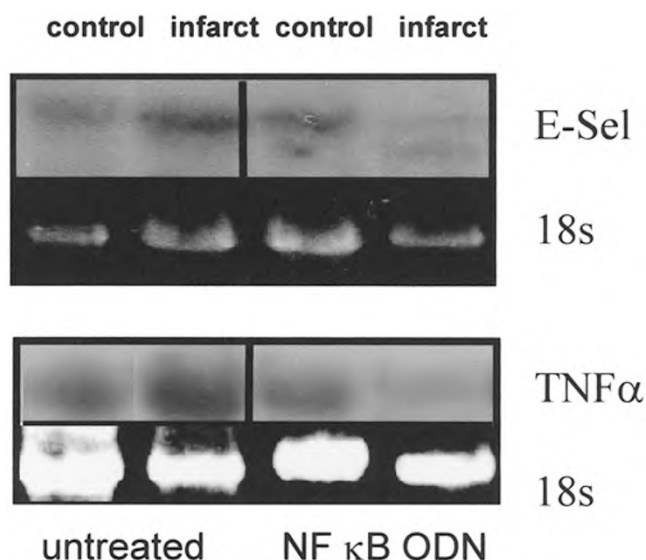


Figure 2 Northern blot of untreated (lane 1–2) and NFκB ODN treated (lane 3–4) hearts using a TNFα probe (upper panel) and a E-selectin probe (lower panel). Induction of TNFα or E-selectin was found in infarct regions of untreated, but not of NFκB-treated hearts. Similar results were found in three independent comparisons of untreated and NFκB decoy ODN-treated hearts in the given regions (control and infarct region).

leukocyte influx (Figure 3) and infarct size (Figure 5a) at 24 h of reperfusion. Because reversible reperfusion injury such as myocardial stunning might occur at 24 h of reperfusion, assessment of stable residual myocardial viability and function was performed after 7 days of reperfusion. At this time point, the combination of NFκB decoy retroinfusion and IB4 administration decreased infarct size (Figure 5c) and improved regional function of the area at risk further than either treatment alone (Figure 6). Thus, the cardioprotection provided by NFκB decoy ODN retroinfusion during myocardial ischemia appears additive to systemic CD18 blockade.

Postischemic myocardial inflammation may be caused by two driving forces: first, rapid post-translational modification of proteins, for example, translocation of P-selectin from endothelial storages¹⁹ or release of PAF²⁰ and preformed TNFα,⁹ and second transcriptional activation of adhesion molecules like ICAM-1 and E-selectin,²¹ cytokines like TNFα⁹ or interleukin-6²² and chemokines, eg MCP-1.²³ Both processes appear to contribute independently to postischemic reperfusion injury: acute leukocyte adhesion has been correlated to myocardial stunning, a rapid functional detriment.²⁴ Delayed leukocyte accumulation causing functional detriment of the myocardium was relevant in experimental protocols, where acute leukocyte adhesion was effectively blocked, whereas subacute endothelial activation was not antagonized.⁶ In our study, 1% PMN were found CD18-positive after IB4 infusion, whereas 50% PMN were CD18-positive at 4 h of reperfusion (data not shown). Although infarct size was reduced by IB4, a significant improvement of regional myocardial function was not obtained after 7 days of reperfusion. These moderate improvements achieved by IB4 resemble experimental data obtained with bolus treatment of a selectin antagonist.⁶

An alternative anti-inflammatory strategy, antagonism of the NFκB activation, was provided by NFκB decoy

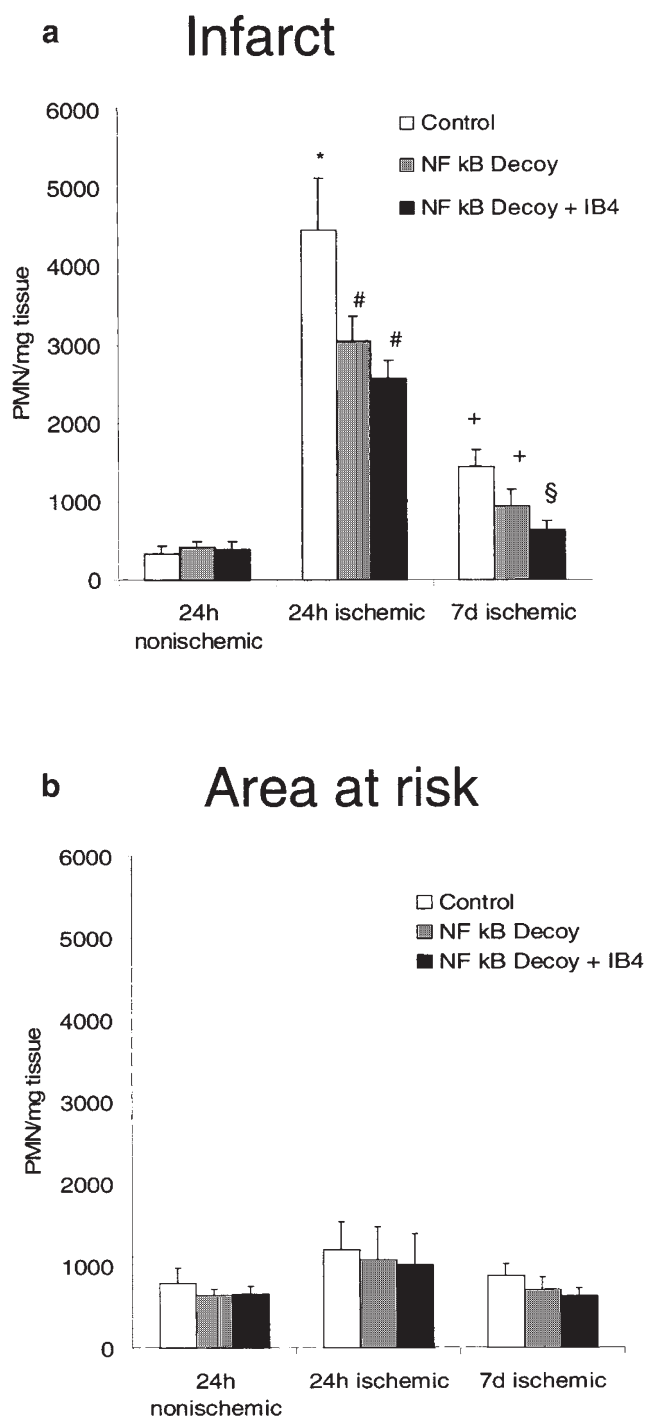


Figure 3 PMN influx into the infarct (a) or area at risk (b) of untreated hearts (open columns) or NFκB decoy (grey columns) or NFκB decoy + IB4-treated hearts (black columns). The inhibitory effect of the NFκB decoy ODN was present only in the infarcted area, whereas no significant effect was found in the area at risk of the treated hearts. Mean \pm s.e.m.; * $P < 0.05$ versus nonischemic tissue; # $P < 0.05$ versus controls at 24 h; + $P < 0.05$ versus 24 h hearts of the same group; § $P < 0.05$ versus controls at 7 days.

ODN transfection. This therapeutic approach has been described first by Morishita *et al.*,¹¹ who reported reduction of infarct size in rats 7 days after postischemic NFκB decoy application. However, coronary artery infusion of NFκB decoy ODN is not feasible in large ani-

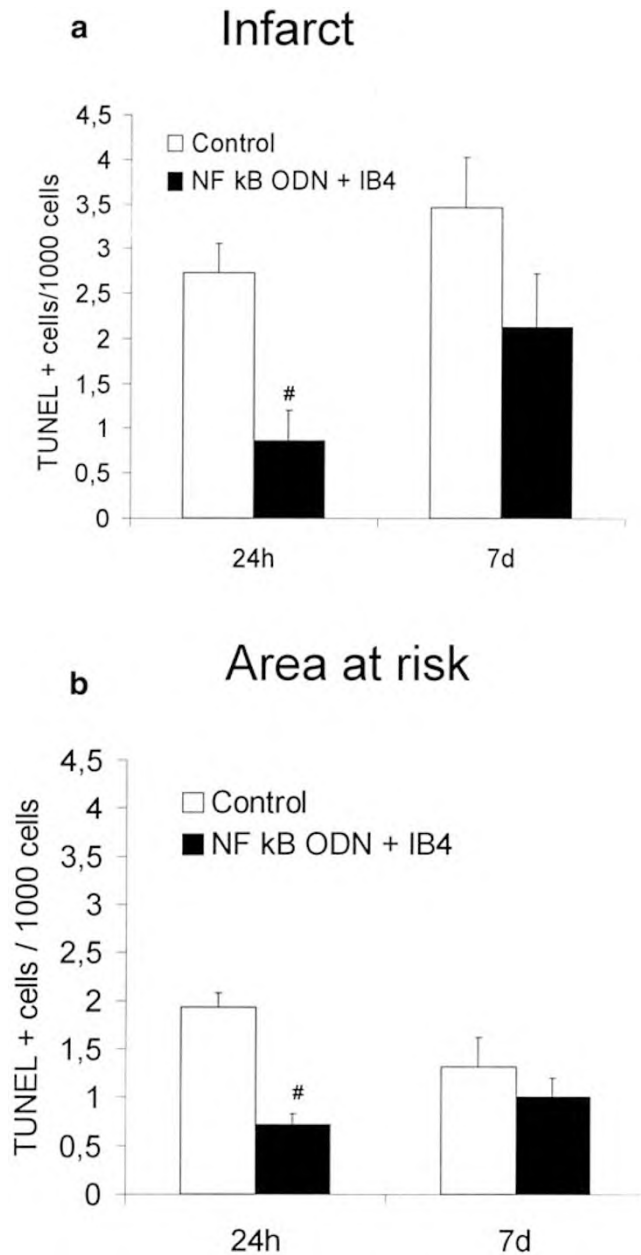


Figure 4 NFκB decoy ODN retroinfusion + IB4 reduces apoptosis. TUNEL-positive cells in the infarcted area (upper panel, controls = open columns) and in the non-infarcted area at risk (lower panel) were reduced by NFκB decoy ODN + IB4 administration (filled columns) at 24 h, but not at 7 days. Mean ± s.e.m.; [#]P < 0.05 versus control.

mals or patients, given the amount of decoy ODN needed, the transfection efficacy and the systemic contamination. In contrast, targeted retrograde delivery of DNA into ischemic myocardium has been successfully used in a previous preclinical study in pigs, providing a 50-fold higher transfection efficacy than antegrade intracoronary transfection.¹⁷ Interestingly, in the present study retroinfusion of the liposome-decoy ODN at the end of the ischemic period sufficed to induce a sustained, though modest cardioprotective effect in our pig model. This effect could be increased by additional CD18 blockade, indicating an independent contribution of acute and

subacute endothelial activation to myocardial ischemia/reperfusion injury.

With regard to side-effects, a potential concern of NFκB decoy oligonucleotide transfection is induction of apoptosis, in particular of myocytes. Cultured myocytes stimulated with TNFα undergo apoptosis at an increased rate, when NFκB is inactivated.¹⁸ As demonstrated in this study, retrograde NFκB decoy ODN transfection was targeted predominantly to the endothelial cell layer (Figure 1b), whereas transfection of the cardiomyocyte compartment appeared low. Moreover, we recently reported²⁵ that apoptosis induced by ischemia and reperfusion in the heart at least in part depends on Fas-L, TNFα and TRAIL, three NFκB-regulated death ligands.^{26–28} In the present study, TNFα mRNA, as well as apoptosis, was found to be reduced in NFκB decoy ODN-treated hearts in comparison to control hearts, suggesting that NFκB decoy ODN application does not result in an increased rate of apoptosis if TNFα expression is not up-regulated.

Does NFκB decoy ODN application pose a risk for cardiac rupture as corticosteroid application did in patients after acute myocardial infarction?^{29,30} Cardiac rupture recently has been demonstrated to depend in part on matrix metalloproteinase activation.^{31,32} Since two proteinases closely related to cardiac rupture, MMP-9 and urokinase plasminogen activator, are transcriptionally regulated by NFκB, decoy inhibition of this transcription factor decreases their expression.^{33,34} On the other hand, NFκB blockade was restricted to the first days of reperfusion, allowing for potential NFκB-dependent repair mechanisms thereafter. Accordingly, in the present study we found one intracardial hemorrhage in a control experiment after 24 h of reperfusion, whereas no NFκB decoy ODN-treated animals suffered from rupture or intramyocardial hemorrhage (data not shown).

In summary, percutaneous transluminal retroinfusion of NFκB decoy ODNs antagonizes postischemic activation of NFκB in the area at risk. Subsequently, PMN recruitment, apoptosis and infarct size were reduced after ischemia and 24 h of reperfusion. After 7 days of reperfusion, infarct size remained reduced. At this time-point, improvement of regional myocardial function in the non-infarcted area at risk was found only if NFκB decoy ODN retroinfusion was combined with anti-CD18 antibody bolus administration. Since selective retroinfusion has been demonstrated to be feasible, safe and efficient in patients,³⁵ a combined anti-inflammatory therapy with CD18 antibody and retrogradely delivered NFκB decoy ODN might be an option for patients with interventional treatment of acute myocardial infarction.

Materials and methods

Materials

The CD18 antibody IB4 was from ATCC. Liposome transfection was performed with Effectene (Qiagen, Hamburg, Germany). Animals were purchased from Oberschleißheim, Germany. All chemicals were from Sigma (Deisenhofen, Germany), if not stated otherwise.

Oligonucleotides and liposomes

Oligonucleotides (ODNs) containing the NFκB consensus binding site (5' AGT TGA GGG GAC TTT CCC AGG C 3' and 5' GCC TGG GAA AGT CCC CTC AAC T 3'), or

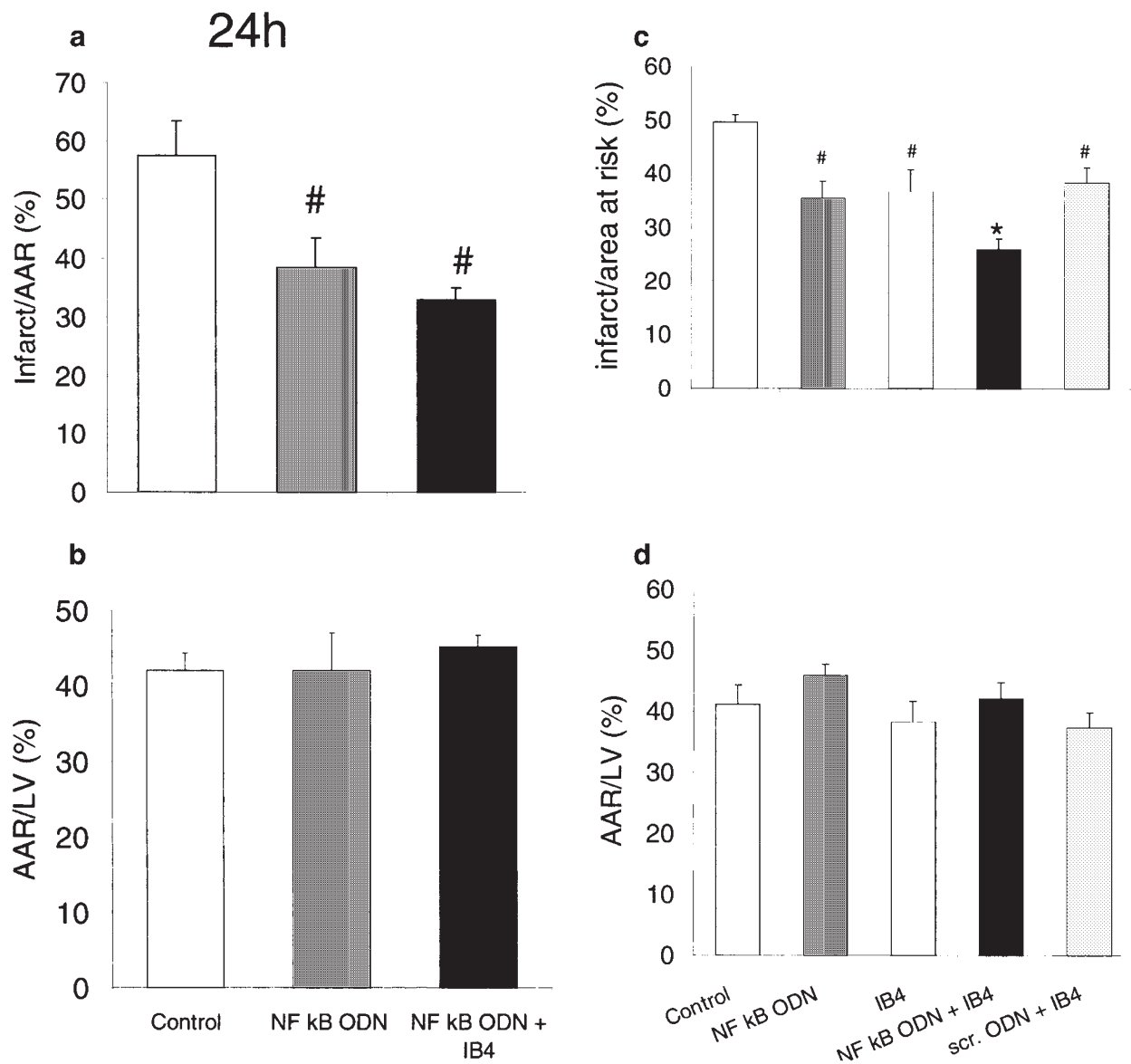


Figure 5 NFκB decoy retroinfusion + IB4 reduces infarct size. At 24 h, NFκB decoy ODN treatment (grey column) reduces infarcted area/area at risk in comparison to control hearts (open columns). NFκB decoy ODN + IB4 treatment did not reveal a greater reduction of infarct size (a, $n = 5$). The area at risk/left ventricular area ratio was comparable in all groups. (b) At day 7, infarct size was reduced by NFκB decoy ODN or IB4, and further decreased with NFκB ODN + IB4 treatment (c; $n = 10$, except IB4 + scrambled ODN with five experiments). Area at risk/LV area did not differ significantly between groups (d). Mean \pm s.e.m.; [#] $P < 0.05$ versus control, ^{*} $P < 0.05$ versus NFκB decoy ODN.

Table 1 Hemodynamic parameters before (pre) and 7d after (post) ischemia and reperfusion

Group	HR		ESP		LVEDP		dP/dt _{max}		dP/dt _{min}	
	pre	post	pre	post	pre	post	pre	post	pre	post
Control	73 \pm 4	78 \pm 3	83 \pm 3	72 \pm 3 ^a	11.8 \pm 0.8	13.4 \pm 0.6	928 \pm 64	825 \pm 53 ^a	-1012 \pm 97	-750 \pm 79
IB4	66 \pm 4	65 \pm 5	80 \pm 2	80 \pm 3	11.8 \pm 0.6	12.5 \pm 1.2	918 \pm 71	869 \pm 79	-971 \pm 45	-872 \pm 63
NFκB ODN	74 \pm 7	71 \pm 3	77 \pm 4	76 \pm 4	10.1 \pm 1	11.0 \pm 0.8	854 \pm 63	901 \pm 40	-926 \pm 69	-863 \pm 54
IB4 + NFκB ODN	75 \pm 5	71 \pm 3	80 \pm 4	80 \pm 2	13.7 \pm 0.8	14.0 \pm 0.7	1006 \pm 89	1072 \pm 81	-1108 \pm 73	-1020 \pm 108

HR, heart rate; ESP, end systolic pressure; LVEDP, left ventricular end diastolic pressure.

^a $P < 0.05$ versus pre-ischemic value.

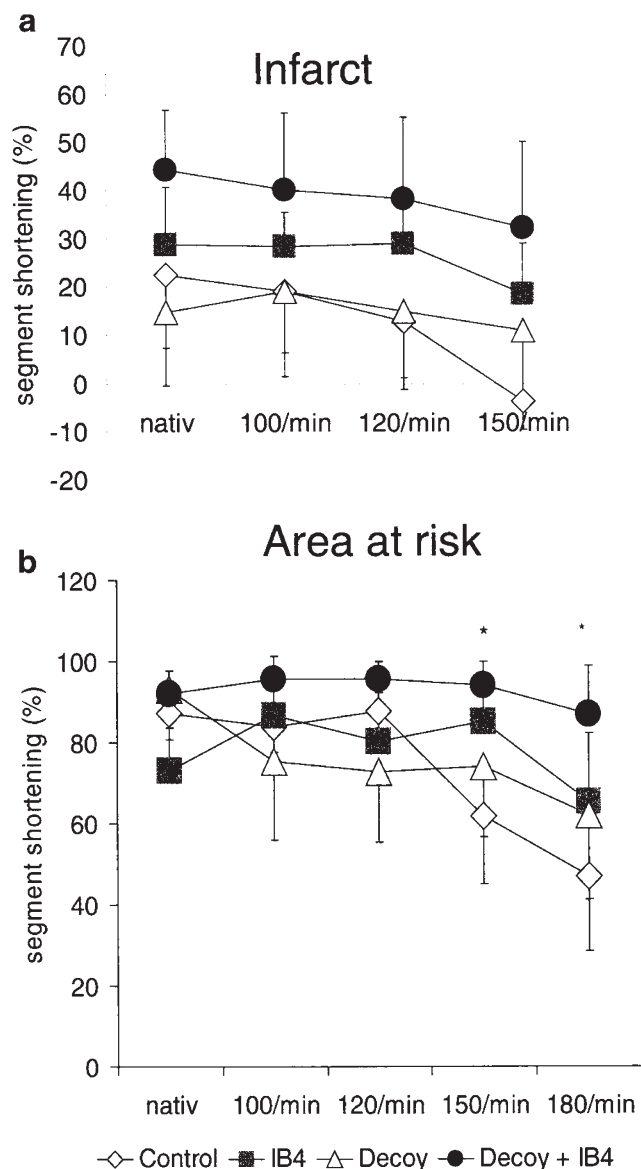


Figure 6 NFκB decoy ODN + IB4 treatment induces improvement of regional myocardial function of the area at risk. (a) Segment shortening in the infarct zone normalized to segment shortening of the Cx perfusion area. No significant differences were detected between groups. (b) Segment shortening in the area at risk normalized to Cx perfusion area. Despite similar segment shortening at rest in all groups, functional reserve at rapid pacing (150–180/min) was greater in NFκB + IB4-treated hearts than in untreated hearts (Mean ± s.e.m.; *, $P < 0.05$ compared with control).

a nonbinding sequence (5' TTG CCG TAC CTG ACT TAG CC 3' and 5' GGC TAA GTC AGG TAC GGC AA 3') were manufactured by MWG (Ebersberg, Germany). Where indicated, NFκB decoy was labeled with digoxigenin or AMCA at the 5' end. 75 nmol of double-stranded NFκB decoy or scrambled ODN were used per heart. Using Effectene (Qiagen), the DNA-liposome mix was produced according to the manufacturer's recommendations, diluted to a volume of 20 ml with NaCl 0.9% and retroinfused over 5 min.

Cell culture experiments

Rat coronary endothelial cells were cultured as previously described.³⁶ Subconfluent cells were transfected with Effectene (Qiagen) containing a construct of firefly luciferase ligated to an NFκB-sensitive promoter of the human ICAM-1 gene (277-ICAM-1)³⁷ and a control construct of renilla luciferase ligated to an NFκB-insensitive tyrosin-kinase promoter. 24 h later, cells were stimulated with TNFα (100 ng/ml). NFκB decoy ODN (2 μg/well) containing liposomes were added either at stimulation (0 h) or 24 h before (-24 h). After stimulation with TNFα, cells incubated for 24 h, before they were harvested and pellets were analyzed for luciferase activity after substrate supplementation according to manufacturer's instructions (Promega, Mannheim, Germany). Results are given as firefly luciferase activity divided by renilla luciferase activity (%).

Animal experimental protocol

The care of the animals and all experimental procedures conform with the German animal legislation and were approved by the local animal protection commission. All pig experiments were conducted at the Institute for Surgical Research of the University of Munich. German farm pigs were anesthetized and instrumented as described previously.³⁸ The external jugular vein and the carotis artery of the right side were cannulated and appropriate sheaths (8F) were placed. Thereafter, measurements with a Miller pressure tip catheter placed in the left ventricle, eg LVEDP, dP/dt_{max} and dP/dt_{min} , were performed (expressed as mean of five cardiac cycles/time point).

Ischemia

A balloon was placed in the LAD distal to the bifurcation of the first diagonal branch, and inflated with 4 at. Correct localization of the coronary occlusion and patency of the first diagonal branch was ensured by injection of contrast agent. At 30 min of ischemia, antibody infusion was performed for 15 min, where indicated.

Retroinfusion

As described in detail previously,³⁹ the retroinfusion catheter (PTC) was advanced into the anterior interventricular vein (AIV) draining the parenchyma perfused by the LAD. After assessment of the individual occlusion pressure of the venous system, retroinfusion pressure was set 20 mmHg above the latter. After 55 min of ischemia, continuous pressure regulated retroinfusion¹⁷ of isothermic NaCl 0.9% (20 ml/min) without or with separate, reflux valve-protected infusion of liposomes containing NFκB decoy or scrambled ODN was conducted. At 60 min, retroinfusion was stopped and suction of the vein was performed for 30 s to prevent ODN dissemination.

Reperfusion

At 60 min of ischemia, after completion of retroinfusion, the balloon in the LAD was deflated. Thereafter, venous and arterial sheaths were withdrawn, the vessels ligated and suture of the muscle and the skin performed. Animals were brought back to the animal facility where they were fed with water and nutrition *ad libitum*.

At the end of the experiment (day 1 or 7 after ischemia) animals were brought back to the OR, anesthetized and instrumented. After hemodynamic measurements, ster-

notomy was performed and the pericardium was removed. Ultrasonic crystals were placed 1 cm (area at risk) and 4 cm distal to the balloon occlusion site. Each pair was inserted with its axis perpendicular to the LAD. Another pair of crystals was inserted in the Cx perfusion area. Sonomicrometry measurements were performed under control conditions, as well as with atrial pacing (80, 100, 120, 150/min for 1 min each), as described before.³⁹

Infarct size determination

Before arrest of the heart, 20 ml of methylene blue were injected in the left ventricle for negative staining of the area at risk. After heart excision, the infarct size was determined by pressure controlled injection (80–100 mmHg) of 15 ml of 10% tetrazolium-red. Thereafter, the left ventricle was cut into slices of 5 mm thickness, which were subjected to digital photography. Image processing of the digital pictures with trace measurements (SigmaScan) provided volume of infarct region, area at risk and total LV area for each slice.⁴⁰

Tissue analysis by fluorescence histology, EMSA, Northern blot and MPO-assay

Efficacy of ODN transfection was assessed by fluorescence microscopy of transfected and control areas. For this purpose, AMCA- or digoxigenin-labeled oligonucleotides were retroinfused during ischemia. At days 1 and 7 of reperfusion, hearts were excised and transfected areas were compared with control areas (Figure 1a). The functional impact of NF κ B decoy transfection was assessed by electromobility shift assays,⁹ myeloperoxidase assay⁴¹ and Northern blot⁹ of target genes E-selectin and TNF α , as described previously. Probes for Northern blotting were derived from cDNA after reverse transcription of pig myocardial mRNA using the following primers: 5'CTG GAG AAG GAT GAT CGA CT 3' and 5'CAG GGA AGT CTG GAA AAT TGG 3' for TNF α , as well as 5'CCA GAG AGA TCA ACA TGA GC 3' and 5'CTG AGA AGA GCC AGA GAC 3' for E-selectin.

Tunel assay

TUNEL staining was performed in ventricular tissue sections as described using DAB.²⁵ Sections were analyzed using color thresholding and size-oriented image processing under stable relative illumination (KS 400 software, Zeiss, Oberkochen, Germany). 500 cells were analyzed from each section, and five sections were analyzed per region (infarct, AAR, control), with five hearts compared per group.

Statistical methods

The results are given as mean \pm s.e.m. Statistical analysis was performed with one way analysis of variance (ANOVA). Whenever a significant effect was obtained with ANOVA, we performed multiple comparison tests between the groups using Student-Newman-Keul's procedure (SPSS statistical program). Differences between groups were considered significant for $P < 0.05$.

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