

# Activation of Nuclear Factor- $\kappa$ B Significantly Contributes to Lumen Loss in a Rabbit Iliac Artery Balloon Angioplasty Model

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**Background**—To investigate the contribution of inflammation to postangioplasty lumen loss, we used an adenoviral gene therapy approach to inhibit the central inflammatory mediator nuclear factor- $\kappa$ B (NF- $\kappa$ B) by overexpression of its natural inhibitor, I $\kappa$ B $\alpha$ .

**Methods and Results**—The adenovirus carrying human I $\kappa$ B $\alpha$  was applied immediately after balloon dilatation by a double-balloon catheter in a rabbit iliac artery restenosis model. Immunohistochemistry of I $\kappa$ B $\alpha$  revealed that mainly smooth muscle cells of the media but also cells of the adventitia were transduced and expressed the transgene I $\kappa$ B $\alpha$  for  $\geq 8$  days. At this time point, intercellular adhesion molecule-1 (30%) and monocyte chemoattractant protein-1 (50%) expression, as well as recruitment of macrophages into the wounded area (90%), were significantly reduced in I $\kappa$ B $\alpha$ -treated vessels. In addition, expression of inhibitor of apoptosis proteins was reduced and the percentage of apoptotic cells was increased compared with control-treated contralateral vessels. Animals killed 5 weeks after treatment exhibited a significantly reduced degree of lumen narrowing ( $P < 0.02$ ) on the side treated with adenovirus I $\kappa$ B $\alpha$ . The lumen gain of  $\approx 40\%$  was due to positive remodeling.

**Conclusions**—From these data, we conclude that balloon angioplasty-induced activation of NF- $\kappa$ B contributes to lumen loss likely via induction of an inflammatory response and a decrease in the rate of apoptosis. These data show for the first time that inflammation mediated by NF- $\kappa$ B is involved in postangioplasty lumen narrowing. Specific and more potent inhibitors of NF- $\kappa$ B might therefore be a useful therapeutic measure to improve clinical outcome after balloon dilatation. (*Circulation*. 2002;105:633-638.)

**Key Words:** restenosis ■ inflammation ■ gene therapy ■ nuclear factor- $\kappa$ B ■ apoptosis

Percutaneous transluminal balloon angioplasty, widely used in the treatment of obstructed atherosclerotic vessels, is hampered by restenosis in  $>40\%$  of patients who initially are treated successfully.<sup>1</sup> The mechanisms implicated in the process of restenosis include growth factor-dependent activation of smooth muscle cell (SMC) proliferation,<sup>2</sup> protease-dependent migration of cells into the wounded area, cytokine-initiated matrix deposition,<sup>3</sup> and progression of the atherosclerotic disease itself.<sup>1</sup> Because inflammation is implicated in the development of atherosclerosis, we hypothesized that it might also contribute to postangioplasty vessel narrowing. The common factor driving the inflammatory response is the transcription factor NF- $\kappa$ B.<sup>4,5</sup> In resting cells, NF- $\kappa$ B is held in the cytosol by complex formation with its natural inhibitor, I $\kappa$ B $\alpha$ .<sup>6</sup> Upon stimulation, eg, by inflammatory cytokines, I $\kappa$ B $\alpha$  is phosphorylated, ubiquitinated, and degraded by the proteasome.<sup>7</sup> NF- $\kappa$ B is in turn liberated and enters the

nucleus to initiate transcription of adhesion molecules,<sup>8</sup> cytokines, and other inflammatory mediators,<sup>4</sup> as well as of the inhibitor of apoptosis proteins (IAP).<sup>9</sup>

Activated NF- $\kappa$ B has been found within human atherosclerotic lesions or after angioplasty<sup>10</sup> but not in normal arteries.<sup>11</sup> The contribution of NF- $\kappa$ B-dependent pathways to restenosis, however, is not known. We used an adenovirus-mediated gene transfer approach of the specific inhibitor of NF- $\kappa$ B, I $\kappa$ B $\alpha$ , to the dilated iliac artery in a rabbit restenosis model to analyze the contribution of NF- $\kappa$ B to the restenosis process.

## Methods

### Adenovirus Constructs

The adenoviral construct for overexpression of I $\kappa$ B $\alpha$  contained the coding sequence for human I $\kappa$ B $\alpha$  together with a nuclear localization signal under the cytomegalovirus promoter (rAd.I $\kappa$ B $\alpha$ ) as described previously.<sup>12</sup> The control adenoviral constructs (rAd.GFP and rAd. $\beta$ -gal), whose ability to transfect SMCs has been described

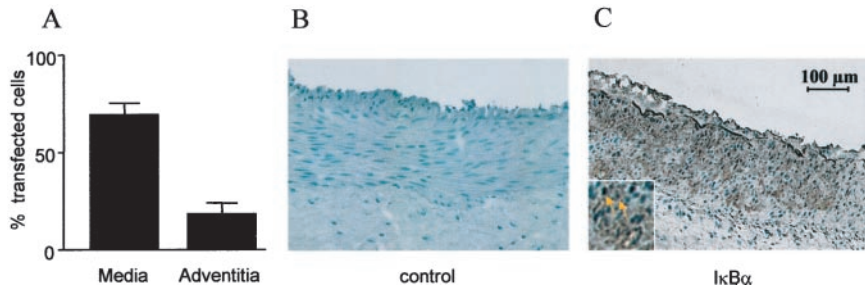
Received September 10, 2001; revision received November 16, 2001; accepted November 16, 2001.

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**Figure 1.** Expression of IκBα transgene in rabbit iliac artery 5 days after adenovirus-mediated gene transfer; adenovirus was applied via double-balloon catheter (1.5 mL of 10<sup>6</sup> pfu/mL; 6 atm pressure) immediately after balloon dilatation. A, Extent of transfection in percent of cells in media and adventitia, respectively. B, Vehicle-treated control vessel. C, IκBα adenovirus-treated vessel; abundant staining of IκBα is seen primarily in SMCs of media. Enlarged inset shows nuclear staining of transgene (orange arrows).

previously,<sup>13</sup> gave results comparable to the vehicle PBS and will be referred to collectively as “control treatment” unless otherwise stated. An adenovirus construct containing the coding sequence for the X-chromosome-linked IAP (rAd.XIAP), also described previously,<sup>14</sup> was used for in vitro apoptosis assays.

**Animal Preparation, Balloon Angioplasty, and Gene Transfer**

Forty New Zealand White rabbits received a diet of rabbit chow supplemented with 1.5% cholesterol and 7% peanut oil for 3 weeks before the experiments. All animal experiments were approved by the governmental committee for animal research.

Animal experiments were performed essentially as described previously.<sup>15</sup> Angiography in digital subtraction mode was performed in all animals before and after balloon dilatation and before animals were killed. In 40 animals, balloon dilatation of both iliac arteries and local drug delivery were performed with a multichannel double-balloon catheter (3-mm diameter, 20-mm length) at 3 atm for angioplasty and 5 to 6 atm for drug delivery with a LeVein inflator with pressure gauge (both from Boston Scientific, Mediatech). Human IκBα-expressing adenovirus (3 mL of 1×10<sup>9</sup> pfu/mL) or control was injected immediately after angioplasty. In all 40 animals, 1 side was treated with rAd.IκBα; on the contralateral control sides, 5 animals received rAd.GFP7, 7 rAd.β-gal, and 28 received the vehicle PBS. Eight animals died of intervention-related causes; the remaining animals were killed in 2 groups, either 5 to 8 days (n=13) or 4 to 5 weeks (n=19) after treatment.

At the time the animals were killed, the iliac arteries were flushed in situ with 10% sucrose in PBS via an inserted 19-gauge Venflon for cryopreservation. The distal aorta and the iliac vessels, including 2 cm distal to the treated segment, were then removed en bloc. Both iliac arteries were cut into 3-mm segments, embedded in optimal cutting temperature medium (OCT; Miles), frozen, and stored at -70°C until use.

**Immunohistochemistry and In Situ Nick Translation**

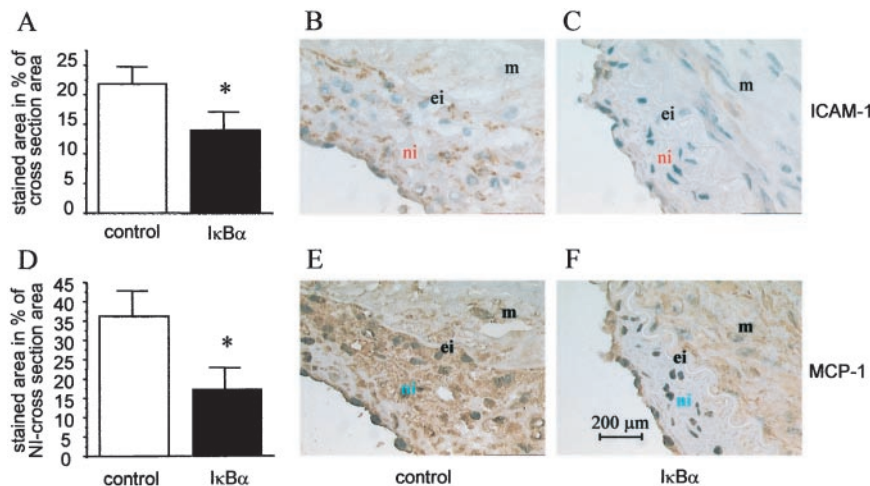
Immunohistochemistry was performed on snap-frozen tissue with either peroxidase- or fluorescence-based procedures. Antibodies used were IκBα antibody sc-371 (Santa Cruz Biotechnology Inc); proliferation antigen Ki67 (Dako); monocyte/macrophage lineage anti-rabbit CD68 and RAM11 (Dako); intercellular adhesion molecule-1 (ICAM-1; monoclonal; a kind gift of Dr Myron Cybulsky) and monocyte chemoattractant protein-1 (MCP-1; polyclonal goat; a kind gift of Dr Akihiro Matsukawa), the staining of which was quantified with AnalySiS software (SIS Soft Imaging Software Corp) with a constant color threshold and which is given in percent of positive vessel cross-sectional area; and XIAP, cIAP<sub>1</sub>, and cIAP<sub>2</sub> antibodies (R&D Systems, Inc). For detection of IAPs, antibodies were preincubated with the peroxidase-labeled secondary antibody at a 2:1 ratio, absorbed with preimmune rabbit serum for 15 minutes, and then used for immunostaining with diaminobenzidine (Dako) for visualization. DNA single-strand breaks were visualized in acetone-fixed tissue sections by the in situ nick translation method.<sup>16</sup> Positive cells were counted in 3 cross sections per vessel segment.

**Histomorphometric Analysis**

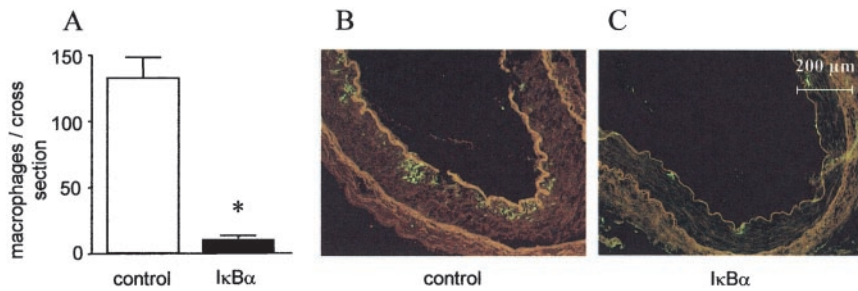
Cross sections of the cut segments of the dilated vessels were assessed histomorphometrically. Images (Olympus AX70 microscope) were frame grabbed and analyzed with AnalySiS software. We measured lumen area, area within the internal elastic lamina, area within the external elastic lamina, neointima area, and number and length of fractures in the internal elastic lamina.

**In Vivo Lumen Size Assessment by Angiography**

Angiograms obtained in digital subtraction angiography mode were analyzed with AnalySiS software. We assessed the lumen of the iliac arteries immediately before treatment and at the time the animals were killed 4 to 5 weeks after treatment and compared the changes



**Figure 2.** Expression of NF-κB-regulated inflammation-related proteins ICAM-1 and MCP-1 in vessel wall 5 days after balloon injury in vehicle or IκBα adenovirus-treated vessels. A, Quantification of ICAM-1 expression after vehicle or IκBα gene transfer. B and C, ICAM-1 expression in iliac artery after control treatment (B) and rAd.IκBα treatment (C). D, Quantification of MCP-1 expression after vehicle or IκBα gene transfer. E and F, MCP-1 expression in neointima of iliac artery after control treatment (E) or rAd.IκBα treatment (F). m indicates media; ei, inner elastic lamina; and ni, neointima.



**Figure 3.** Recruitment of monocytes/macrophages into vessel wall 5 days after balloon injury followed by application of vehicle or IκBα adenovirus. A, Number of recruited macrophages was reduced by 90% ( $P < 0.0001$ ). Immunohistochemical detection of macrophages in sections from vehicle (control)-treated side (B) and adenovirus IκBα-treated side (C).

between the 2 time points for the treated and contralateral control-treated arteries.

### In Vitro Apoptosis Assay

Subconfluent cultures of human artery SMCs<sup>13</sup> were either used untransfected or were transfected with rAd.IκBα or rAd.IκBα together with rAd.XIAP and exposed to tumor necrosis factor-α (TNF-α; 100 U/mL) for 16 hours, essentially as described previously for endothelial cells.<sup>14</sup> Thereafter, apoptosis was assessed by counting numbers of attached and detached apoptotic cells as described previously<sup>17</sup>; with fluorescence-activated cell sorter analysis was used to ensure that detached cells were apoptotic.

### Statistical Analysis

Data are given as mean ± SEM. Statistical differences were calculated by ANOVA or paired Student *t* test as indicated. Data were considered significant if a *P* value of <0.05 was reached.

## Results

### IκBα Is Expressed After Adenovirus-Mediated Gene Transfer

In all samples obtained 5 to 8 days after intervention, the transgene was detected throughout the media ( $69.4 \pm 5.7\%$  of the cells) and in the adventitia ( $19 \pm 4.9\%$ ) of the balloon-treated vessels (Figure 1), whereas in peripheral tissues, only small foci in liver and rare foci in kidney and lung were seen.

### Effect of IκBα Overexpression on Inflammatory Response

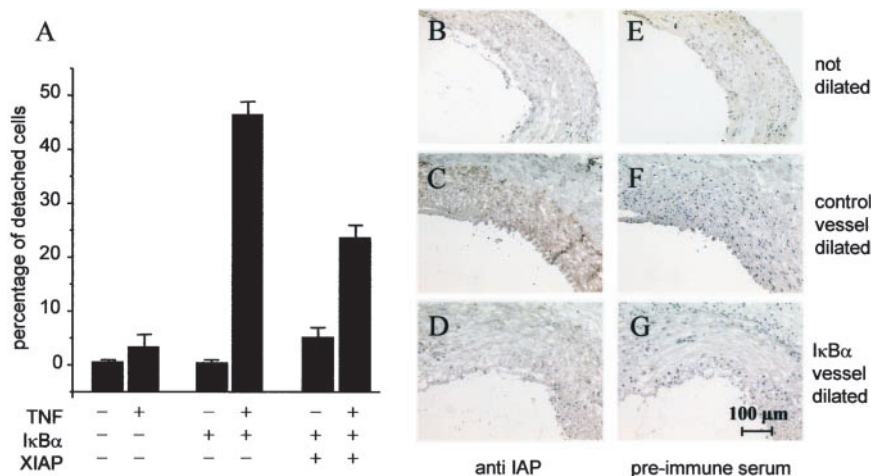
In animals killed 5 to 8 days after intervention, both ICAM-1 and MCP-1 staining were significantly reduced in rAd.IκBα-treated arteries versus the respective control arteries by 48%

and 35%, respectively (Figure 2). The number of cells that stained positive for monocyte lineage was consistently and highly significantly lower on the IκBα-treated side than on the control side. On average, recruitment of macrophages was reduced by 91% ( $P < 0.0001$ ) in balloon-dilated vessel segments treated with the IκBα-coding adenovirus compared with control vessel segments (Figure 3).

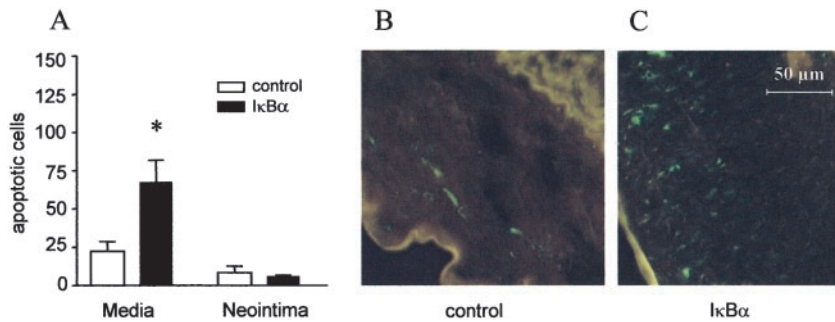
### Effect of IκBα Overexpression on Apoptosis

In tissue sections obtained 5 to 8 days after balloon dilatation, an upregulation of IAPs in control arteries could be demonstrated (Figure 4, B and C), whereas in rAd.IκBα-treated arteries, such upregulation was not seen (Figure 4, C and D). Consistently, the number of apoptotic cells on the treated side was almost 3 times the number on the control side (Figure 5). Apoptotic cells were found mainly in the media of the vessel wall.

To prove whether reduction in NF-κB-induced upregulation of IAPs by rAd.IκBα influenced the susceptibility of SMCs toward TNF-α-induced apoptosis, we employed an experimental protocol used by us previously to answer a similar question for endothelial cells.<sup>14</sup> When subconfluent<sup>18</sup> SMCs were incubated with 100 U/mL TNF-α for 16 hours, fewer than 5% of cells were found to be apoptotic; this percentage increased to almost 50% in rAd.IκBα-treated cells (Figure 4A). When SMCs were transduced with both rAd.IκBα and rAd.XIAP, the number of apoptotic cells increased on TNF-α treatment to ≈25%, which indicates partial reversion of the rAd.IκBα effect.



**Figure 4.** Effect of IκBα overexpression on apoptosis. A, XIAP overexpression rescues IκBα-overexpressing cells from TNF-α-induced apoptosis. Cultured umbilical artery SMCs transfected with either rAd.IκBα alone or rAd.IκBα and rAd.XIAP together were treated with TNF-α, and percentage of detached apoptotic cells was determined. Whereas IκBα-overexpressing cells became susceptible for TNF-α-induced apoptosis, double-transfected cells were partially rescued. B, C, and D, Immunohistochemical detection of IAPs in rabbit iliac arteries with and without IκBα overexpression. B, Minimal expression of IAPs in cells of media above balloon-dilated area. C, Upregulation of IAP expression in cells of media within balloon-dilated area. D, Suppression of IAP upregulation in cells of media after balloon dilatation in rAd.IκBα-treated vessels. E, F, and G, Staining controls omitting specific IAP antibodies.



**Figure 5.** Apoptosis in vessel wall 5 days after angioplasty and gene transfer is increased in  $I\kappa B\alpha$ -treated vessels. A, Number of cells per cross section that stained positive for apoptosis (mean  $\pm$  SD, terminal dUTP nick end-labeling staining) was significantly increased ( $*P < 0.05$ ) in media but not neointima of adenovirus  $I\kappa B\alpha$ -treated arteries. Examples from 1 rabbit of cross-section from vehicle (control)-treated side (B) and adenovirus  $I\kappa B\alpha$ -treated side (C).

### $I\kappa B\alpha$ Overexpression Does Not Influence the Rate of Cell Proliferation

When sections were stained for proliferating cells, the number of mitotic cells was not significantly different between the  $I\kappa B\alpha$ -treated side and the control side (data not shown). In both vessels, control and  $I\kappa B\alpha$  treated, mitotic cells were found throughout the media and in the adventitia.

### $I\kappa B\alpha$ Overexpression Results in Positive Remodeling and Lumen Gain

In animals killed 5 to 8 days after intervention, neither the lumen area nor dimensions of the vessel layers were different between control- and  $I\kappa B\alpha$ -treated sides. As revealed by angiography 4 to 5 weeks after intervention and immediately before the animals were killed, lumen loss on the control-treated side was on average 40.1%, whereas lumen loss on the rAd. $I\kappa B\alpha$ -treated side was significantly lower ( $P < 0.016$ ), averaging only 22.9% (Figure 6). Therefore, rAd. $I\kappa B\alpha$  treatment resulted in a 42.5% reduction of vessel narrowing. Histomorphometrically, lumen gain was also  $40.3 \pm 2.6\%$  ( $P < 0.025$ ), whereas the area of the neointima and the area of the media were not significantly different between control- and  $I\kappa B\alpha$ -treated vessels. Consistently, vessel size, as assessed by measurement of the area within the external elastic lamina, of  $I\kappa B\alpha$ -treated vessels was increased  $35 \pm 5.6\%$  ( $P = 0.02$ ) over the size of control-treated vessels. Thus,  $I\kappa B\alpha$  overexpression resulted in increased patency of the vessels (Figure 7).  $I\kappa B\alpha$  overexpression did not change the number or length of elastic lamina fractures. Also, the number of elastic lamina fractures did not change from day 5 to 5 weeks

after treatment. Morphometric data are summarized in the Table. Furthermore, orcein staining of elastic matrix proteins did not yield significant differences between rAd. $I\kappa B\alpha$ -treated vessels and control-treated vessels (data not shown).

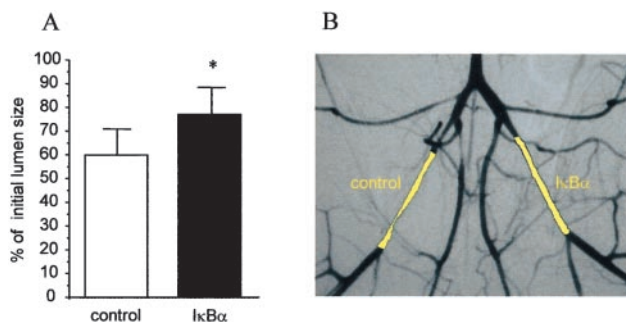
### Discussion

Inflammation appears to be one important mechanism for the development of atherosclerosis: atherosclerotic plaques contain monocytes that secrete mediators such as basic fibroblast growth factor, platelet-derived growth factor, transforming growth factor- $\beta$ , or MCP-1<sup>19</sup>; inflammatory-response genes like vascular cell adhesion molecule-1, ICAM-1, or E-selectin are upregulated<sup>4,20</sup>; and slightly increased levels of plasma C-reactive protein in patients with atherosclerotic disease reflect the ongoing inflammatory process.<sup>21</sup>

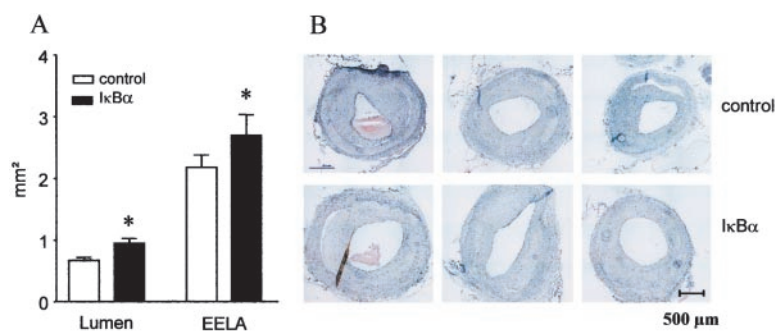
To prove the concept that NF- $\kappa B$ -mediated inflammation also contributes to the restenosis process, we used adenovirus-mediated local overexpression of  $I\kappa B\alpha$  in the balloon-dilated artery, where the transgene was detectable for  $\geq 8$  days. This time frame is consistent with adenovirus-mediated gene expression of other proteins for which expression for up to 2 to 3 weeks has been reported.<sup>22</sup> Because of the specific use of a double-balloon catheter, expression of the transgene was almost exclusively local. Local transduction efficiency was high because of the absence of major concentrations of serum, owing to the delivery of the vector in PBS, so that even cells of the adventitia were transduced. Therefore, the method applied for transduction resulted in high expression of the transgene throughout the vessel wall at the site of treatment.

Transduction resulted not only in expression of the transgene but also in functional inhibition of NF- $\kappa B$  and in turn a reduced inflammatory response as evidenced by ICAM-1 and MCP-1 staining. In the balloon-dilated vessel wall, the number of cells that stained positive for macrophage lineage was consistently reduced by rAd. $I\kappa B\alpha$  treatment to almost the level of uninjured vessels. Activation of the NF- $\kappa B$  system was seen in a rat carotid artery balloon injury model<sup>11</sup> previously, but here we show that inhibition of the NF- $\kappa B$  pathway by overexpression of  $I\kappa B\alpha$  results in a significantly diminished inflammatory response.

Others have reported that adenovirus-mediated gene transfer results in a local inflammatory response. In the present study, this was not seen, because the transgene itself would not only block an exogenously induced



**Figure 6.** Angiographic assessment of changes in lumen size from before treatment to 4 to 5 weeks after treatment. A, Quantification of remaining lumen size of rAd. $I\kappa B\alpha$ -treated arteries compared with contralateral control-treated arteries. B, Example of angiogram 4 weeks after control treatment and rAd. $I\kappa B\alpha$  treatment, respectively.  $*P < 0.05$  vs control.



**Figure 7.** Morphometric analysis of vessel walls 4 to 5 weeks after angioplasty and adenoviral gene transfer. **A**, Mean values and SEM for areas (mm<sup>2</sup>) of lumen and external elastic lamina (EELA) of vehicle-treated arteries (control) and adenovirus IκBα-treated arteries (IκBα). Lumen area was significantly larger in vessels treated with IκBα adenovirus than in vehicle-treated vessels (lumen gain of ≈40%; \**P*<0.025). In addition, area of external elastic lamina was also significantly larger in IκBα-treated arteries (≈35%, \**P*=0.02). **B**, Examples from 1 rabbit of cross-sections from vehicle (control)-treated side (top) and adenovirus IκBα-treated side (bottom).

inflammatory response but also endogenous activation of NF-κB by the adenovirus.<sup>23</sup> From these data, we conclude that transduction of the vessel wall with the adenoviral construct overexpressing IκBα resulted in effective inhibition of the local inflammatory response for ≥8 days.

Activation of NF-κB not only results in upregulation of inflammatory-response proteins but also in upregulation of IAPs.<sup>8</sup> These IAPs have been shown by us and others<sup>14</sup> to inhibit apoptosis caused by TNF-α, and that caused by the anoikis (loss of matrix contact) process.<sup>24</sup> Therefore, inhibition of NF-κB would result in a significantly increased rate of apoptosis. In fact, we could show that balloon dilatation resulted in an upregulation of IAPs that was almost completely inhibited in arteries treated with rAd.IκBα. The number of apoptotic cells in the media was consistently and highly significantly increased in the IκBα-treated vessel compared with control vessels. Such an effect of inhibition of the NF-κB pathway on apoptosis in SMCs was described previously by us.<sup>18</sup> In the present study, we extend these *in vitro* data to *in vivo* effects. We furthermore show that inhibition of upregulation of IAPs in the course of the inflammatory response is responsible for the increased rate of apoptosis, because simultaneous overexpression of 1 of the IAPs by recombinant adenovirus gene transfer of XIAP partially inhibited the increase in apoptosis in SMCs transfected with IκBα. This partial rescue is likely explained by the fact that we overexpressed only 1 of the 3 IAPs.

In contrast, the rate of proliferation as visualized by antibodies staining for the proliferation antigen Ki67 was not significantly different between IκBα-treated sites and control sites. Although Bellas et al<sup>25</sup> showed that NF-κB is necessary for complete proliferation of SMCs, inhibition of the NF-κB pathway for ≈1 week by rAd.IκBα was not sufficient in our experiments to significantly influence proliferation. However, we cannot exclude that such activities are involved in the overall effects seen with

rAd.IκBα transfection. Taken together, overexpression of IκBα likely resulted in inhibition of NF-κB activity not only with respect to inflammatory-response genes but also with respect to other NF-κB-dependent genes.

Such inhibition of NF-κB in the first weeks after injury resulted in a significant lumen gain after 4 to 5 weeks, as evidenced by *in vivo* angiography and morphometry. The angiography data ensured that the changes seen by morphometry were not influenced by differences in the fixation process. The main cause of lumen gain in the present study was an increase in vessel diameter. In contrast, the size of the neointima was not significantly different. Such a process of favorable remodeling might be due to the increased rate of apoptosis in the media during the time when NF-κB is inhibited.

From these data, we conclude that NF-κB contributes to angioplasty-induced lumen loss, likely via induction of an inflammatory response and a decreased rate of apoptosis. Inhibition of the inflammatory response and inhibition of NF-κB-dependent upregulation of IAPs immediately after balloon injury by overexpression of the NF-κB inhibitor IκBα with an adenoviral vector can reduce lumen loss by more than one third. Our data not only show for the first time that NF-κB is involved in postangioplasty lumen loss but might also offer an additional explanation for the beneficial effect of the nonspecific antiplatelet drug aspirin (acetylsalicylic acid), because aspirin inhibits not only platelet aggregation but also NF-κB.<sup>26</sup> Specific and more potent inhibitors of NF-κB might therefore become a useful tool to improve the clinical outcome after balloon dilatation.

### Acknowledgments

This study was supported in part by the ICP Program of the Austrian Federal Ministry for Education, Science and Culture; the Austrian Science Foundation grant No. SFB F509; and a grant from CIRSE. We also would like to acknowledge the excellent technical assistance of Thomas Nardelli in the preparation of the manuscript.

### Effects of IκBα Gene Transfer on Histomorphometric Parameters 4 to 5 Weeks After Treatment

	Lumen, mm <sup>2</sup>	NI, mm <sup>2</sup>	NI/M	Injury Index, FL/FL+IEL	EEL, mm <sup>2</sup>	IEL, mm <sup>2</sup>	EEL, mm	IEL, mm	5–8 Days	4–5 Weeks
Control	0.61±0.04	0.93±0.14	1.15±0.18	0.028±0.014	2.23±0.035	1.54±0.26	5.36±0.51	4.439±0.34	13 (2,3,8)*	19 (3,2,14)*
IκBα	0.87±0.07†	0.94±0.24	1.03±0.12	0.033±0.015	2.68±0.21	1.81±0.16	5.78±0.34	4.774±0.18	13	19

NI indicates neointima; M, media; FL, fracture length; EEL, externa elastica lamina; and IEL, internal elastic lamina.

\*Control vehicle subgroups were (rAd.GFP, rAd.βgal, PBS).

†ANOVA *P*<0.05 for rAd.IκBα-transfected vs untransfected control iliac arteries.

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