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LABORATORY INVESTIGATION

Nuclear Factor-κB is Activated in Filter-Implanted Vena Cava

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Abstract

Background Implantation of a retrievable vena cava filter (VCF) is an effective method for preventing pulmonary embolism. Retrieval of filters, however, may be difficult due to intimal hyperplasia and inflammation in the cava wall. The transcription factor nuclear factor-kappaB (NF-κB) plays an important role in regulation of numerous genes participating in the inflammatory and proliferative responses of cells. The present study was to determine whether VCF implantation resulted in activation of NF-κB in the venous neointima.

Methods Filters were placed in vena cava (VC) in four swine for 30 days and then removed. Intimal specimens adhering to the filter struts were analyzed with reference to normal VC tissues. Immunohistochemical analyses were used to assess the NF-κB subunits p65 and p50 and the phosphorylated inhibitor of κB-α (phosphor-IκB-α) in the tissues. NF-κB DNA-binding activity was measured with enzyme-linked immunosorbent assay.

Results As compared to normal VC tissues, the intimal tissues contained higher percentages of cell nucleus-located p65 and p50, and NF-κB DNA-binding activity. Elevated immunoreactivities of p65, p50 and phosphor-IκB-α were also present in the intima.

Conclusion The present study demonstrates for the first time that VCF implantation caused NF-κB activation in neointima. We further demonstrate the activation is at least partly due to phosphorylation of IκB-α. Our data suggest that NF-κB activation would significantly contribute to development of intimal hyperplasia and inflammation in filter-inserted vena cava walls. NF-κB might be a

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therapeutic target for inhibiting filter-induced neointima and improving filter retrieval.

Keywords Vena cava filter · Intima · NF-κB · IκB-α

Introduction

Implantation of vena cava filter (VCF) is effective in the prevention of fatal pulmonary embolism [1]. However, long-term implantation of filters may cause severe complications [1]. Retrieval is imperative to reduce risks of long-term complications [1]. Previous studies including ours have suggested that the major causes of filter retrieval failure were intimal hyperplasia and inflammation in the cava wall [2–6]. Minimizing the vascular responses would potentially improve filter retrieval and reduce the risks caused by long-term implantation [6]. Exploration of efficient therapy methods necessitates studies on molecular and cellular mechanisms underlying these histological changes, which are currently unknown.

The transcription factor nuclear factor-κB (NF-κB) has a well-established role in the inducible regulation of a range of genes controlling the proliferative and inflammatory responses of cells [7, 8]. NF-κB is a heterodimer mainly consisting of the subunits p50 and p65 (RelA). In most unstimulated conditions, the dimer is present in the cytoplasm and remains inactive since it is complexed to one of a family of inhibitory proteins, IκB-α. IκB-α keeps NF-κB in the cytoplasm by obstructing the recognition of the NF-κB nuclear localization signal [7, 8]. When cells are exposed to certain stimuli including reactive oxygen species, proinflammatory cytokines and thrombin, IκB is phosphorylated by IκB kinases and undergoes rapid turnover [7, 8]. IκB phosphorylation thereby triggers the nuclear translocation of NF-κB. In the nucleus, the transcriptional factor binds to the κB DNA regulatory sites and induces expression of target genes that encode proinflammatory and cell cycle regulatory factors [7, 8]. Previous studies have shown that molecular and pharmacological inhibitors of NF-κB suppressed proinflammatory cytokine expression and hyperplasia in both injured arteries and cultured vascular smooth muscle cells [9, 10].

Our goal is to understand mechanisms underlying VCF-induced neointima hyperplasia and to define therapeutic targets for improving VCF retrieval. In the present study, we tested a hypothesis that VCF insertion would induce activation of NF-κB in vena cava neointima caused by filter implantation. To this end, we collected intimal tissues after VCFs were inserted into swine venae cavae. Events associated with NF-κB activation, namely IκB-α phosphorylation, NF-κB nuclear translocation and DNA-binding activity, in the intima tissues were characterized with immunological and immunocytological methods. The characterization was conducted with a reference to normal vena cava tissues.

Materials and Methods

Materials

Unless otherwise stated, all materials were from Sigma (St. Louis, MO). The retrievable OptEase VC filters were from Cordis Endovascular (Miami, FL). Polyclonal antibody to NF-κB p65 and p50 was purchased from Santa Cruz Biotech (Santa Cruz, CA). Monoclonal antibody to phospho-IκB-α was purchased from Cell Signaling (Danver, MA). Texas red-conjugated anti-rabbit-IgG, Texas red-conjugated anti-mouse-IgG and fluorescein-conjugated anti-rabbit-IgG were purchased from Vector Labs (Burlingame, CA).

Placement and Removal of Vena Cava Filter

Animal experimentations including surgery, placement and retrieval of vena cava filters were conducted in swine as we previously described [2, 3]. Briefly, Yorkshire cross swine (80–113 kg) underwent general anesthesia with Telazol. The OptEase Filters were placed in the infrarenal vena cava (VC) via femoral vein under fluoroscopic guidance. Thirty days after VCF implantation, the filter struts were totally covered by intima. The VCFs were then removed via autopsy to obtain the neointima for analysis [2, 3]. Normal tissues were obtained from areas at a distance away from the implantation site of each vena cava [2]. Immediately after filter removal, the tissues adherent to the struts were harvested with forceps. Tissues were either subjected for protein extraction or embedded in OCT compound and rapidly frozen in liquid nitrogen.

To detect whether NF-κB was activated in intima from filtered vena cava (IFVC), intimal tissues were obtained from filter struts and sectioned. For comparison, normal vena cava tissues (NVC) were also collected from areas at a distance from the filter implantation site of each corresponding vessel.

Immunohistology and Image Analysis

Immunohistology and the respective image analysis were conducted as described [7, 11–14]. Detailed description of these techniques can be found in the "Supplementary online material".

Nuclear Extraction and NF-κB DNA-Binding Activity Assay

The assay procedure was described previously [10]. Briefly, vena cava tissue specimens were homogenized with a tissue homogenizer in ice-cold hypotonic lysis buffer. Tissue debris was then excluded from the homogenates by centrifugation. Next, nuclear proteins were extracted with the Nuclear Extraction kit from Active Motif (Carlsbad, CA). Protein concentrations in samples were quantitated with a Bio-Rad protein assay kit. Equal amounts of the nuclear extracts were used to assess NF-κB DNA-binding activity with the TransAM NF-κB ELISA kit (Active Motif) according to the manufacturer's guidelines.

Statistical Analysis

Results are expressed as mean ± SE ($n = 4$). Paired data were evaluated by Student's t test. P values < 0.05 were considered to be statistically significant.

Results

Expression and Nuclear Translocation of NF-κB Subunits p65 and p50 in Tissues of Intima Caused by Implantation of Vena Cava Filters

To detect whether NF-κB was expressed and activated in IFVC and NVC, indirect immunofluorescent analysis was employed to detect expression and nuclear translocation (an index of activation) of NF-κB subunits p65 and p50. As shown in Fig. 1, p65 immunoreactivities, indicated by red fluorescence, were stronger in IFVC than in NVC. When p65 immunofluorescent images were overlaid on DAPI-stained images, we found that p65 was expressed in both cytoplasm and nuclei. As shown in the bar graph of Fig. 1, percentage of nuclei-located p65 was significantly higher in IFVC tissues than in the NVC ($P < 0.05$), suggesting that filter implantation promoted nuclear translocation of NF-κB subunit p65. Similar results were found with immunofluorescent staining of NF-κB subunit p50 as shown in Fig. 2. These results indicated activation of NF-κB in IFVC.

Presence of High NF-κB DNA-Binding Activity in Intimal Tissues Adherent to Vena Cava Filter Struts

It is known that NF-κB transactivation and functioning are through translocation of free NF-κB (p65 and p50) into the nucleus followed by binding to NF-κB DNA regulatory sites [7, 8]. We then conducted experiments to quantitate

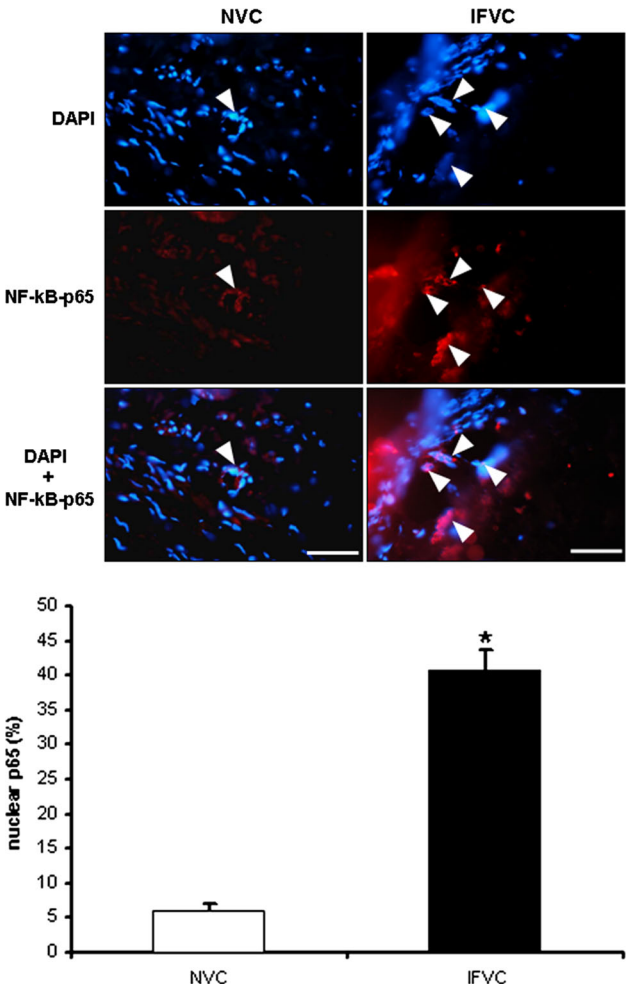

Fig. 1 Expression and nuclear translocation of NF-κB subunit p65 in IFVC versus NVC. IFVC and NVC were collected and sectioned as described in "Materials and Methods". Expression of NF-κB p65 in the tissues was identified by immunofluorescent staining (red) of tissue sections with p65 antibodies. The sections were stained with DAPI (blue color) to visualize nuclei. The staining was analyzed with a fluorescence microscope. Nuclear p65, an index of activation, is indicated by a purple-colored reaction product, and exemplified by arrows in images. The percentage of cells with p65 accumulated in the DAPI-stained nuclei was calculated and is shown in the bar graph. Data are mean ± SE. *$P < 0.05$ versus NVC

NF-κB DNA-binding activity in nuclear extracts of vena cava tissues with a NF-κB ELISA kit, in which oligonucleotide containing the NF-κB consensus site was immobilized onto a 96-well plate. As shown in Fig. 3, these experiments showed that the NF-κB activity was significantly higher in IFVC tissues than in the NVC ($P < 0.05$), further confirming activation of NF-κB by implantation of vena cava filters.

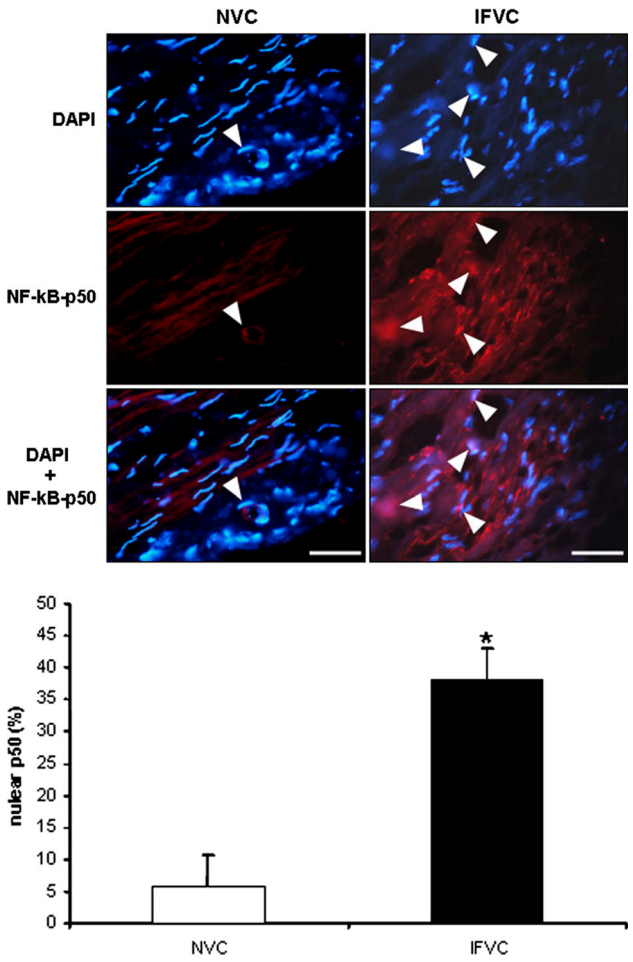

Fig. 2 Expression and nuclear translocation of NF-κB subunit p50 in IFVC versus NVC. Tissues were collected, sectioned and immunohistologically stained as described in "Materials and Methods" section and Fig. 1. Immunofluorescent staining (red) of NF-κB p50 in tissue sections was conducted with antibodies to p50. DAPI-stained nuclei (blue color). Nuclear localization of p50 is indicated by a purple-colored reaction product and exemplified by arrows in images. The percentage of cells with p50 expression in the DAPI-stained nuclei was calculated and is shown in the bar graph. Data are mean ± SE. $^*P < 0.05$ versus NVC

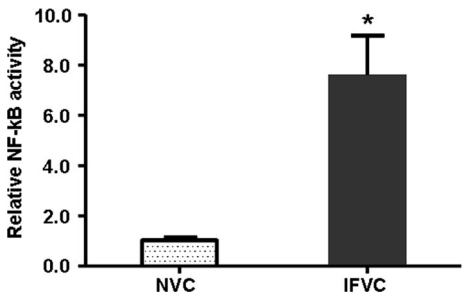

Fig. 3 Presence of NF-κB DNA-binding activity in IFVC versus NVC. Both IFVC and NVC tissues were collected as described in "Materials and Methods" section. Nuclei extracts were prepared from the tissues. NF-κB DNA-binding activity in equal amount of the nuclear extracts was quantitated with an ELISA kit. Relative NF-κB activity in IFVC versus NVC is shown in the bar graph of Fig. 3. Data are mean ± SE. $^*P < 0.05$ versus NVC

Highly Phosphorylated IκB-α Present in Intimal Tissues (IFVC) Adherent to Vena Cava Filter Struts

Classically, the nuclear translocation and activation of NF-κB is through phosphorylation of cytoplasmic IκB-α [7, 8, 15]. To investigate whether IκB-α phosphorylation is present in the sections of the vena cava tissues, we conducted immunofluorescent microscopy with antibodies against phosphorated IκB-α. As shown in Fig. 4, pronounced immunoreactivities of phosphorylated IκB-α were seen in IFVC sections while minimal was present in NVC sections. The immunoreactivities of phosphorylated IκB-α were quantitated with image analyses. The quantitative difference between phosphorylated IκB-α in IFVC sections and in NVC section is depicted in the bar graph of Fig. 4. The results, therefore, indicate implantation of vena cava filters caused significant phosphorylation of IκB-α in IFVC.

Discussion

In the present study, the vena cava filters were removed via autopsy to obtain the neointima for analysis [2, 3]. Normal tissues were obtained from areas at a distance away from the implantation site of each vena cava [2]. We have analyzed and compared NF-κB and IκB systems in NVC and IFVC derived from the same vessel. The main original findings reported in the present study are that intimal specimens from IFVC contained enhanced NF-κB activity as indicated by nuclear translocation and DNA-binding activity when compared to the NVC. We further disclose that pronounced phosphorylation of IκB-α, a canonical upstream event of NF-κB nuclear translocation, was concurrently present in intimal specimens from IFVC. Our data thus suggest that filter implantation increases NF-κB DNA-binding through a mechanism involving enhancing phosphorylation of its inhibitor and then its nuclear translocation. These findings and the corresponding implications are summarized in Fig. 5 and discussed below.

NF-κB activation is usually characterized by analyzing its DNA-binding activity. Nuclear transfer of NF-κB subunits, a relatively indirect event, is also commonly used to assess the activation. Previous studies have documented the presence of constitutive NF-κB activity in normal arteries and arterial cell cultures [8, 10, 16]. The present study shows the presence of constitutive NF-κB expression and activity at low levels in normal vena cava, which extends previous findings on constitutive NF-κB from the artery to

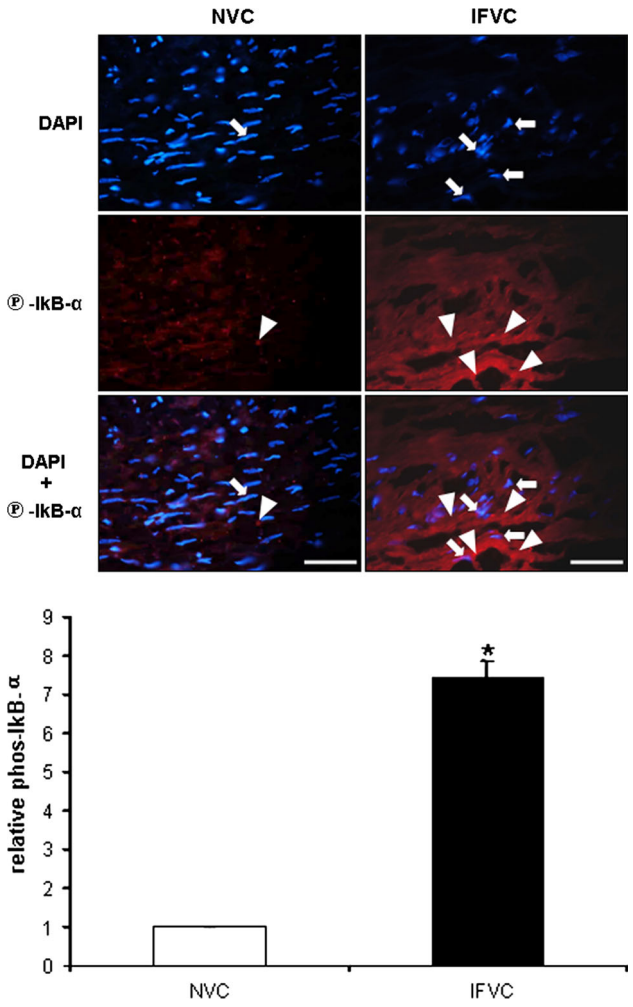

Fig. 4 Phosphorylation of IκB-α present in IFVC versus NVC. Tissues were collected and sectioned as described in "Materials and Methods" section. Tissue sections were subjected to immunofluorescent staining for phosphorylated (p-)IκB-α (red) with antibodies against phospho-IκB-α. Cellular nuclei of all sections were counterstained with DAPI (blue color). Remarked immunoreactivities of p-IκB-α were present in IFVC, while little was found in NVC. Immunofluorescent intensity of p-IκB-α in sections of IFVC and NVC was quantitated as described in "Materials and Methods" section and is shown in the bar graph. Data are mean ± SE. $^*P < 0.05$ versus NVC

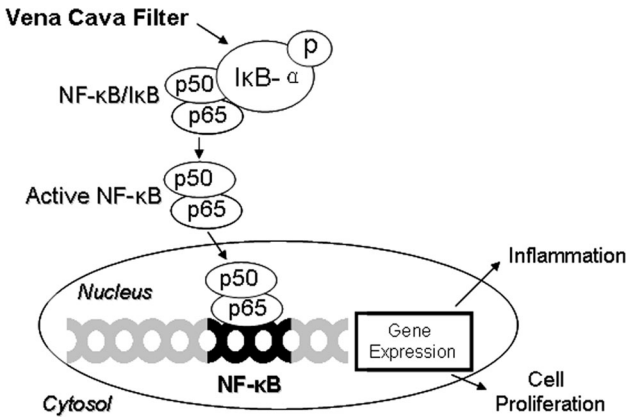

Fig. 5 Schematic diagram of NF-κB activation involved in vena cava filter-induced intima. Vena cava filter implantation causes proinflammatory activation and hyperplasia of neointima overlaying the device struts. During development of the venous neointima, IκB-α in the intimal cells is phosphorylated, followed by translocation of free NF-κB subunits p65 and p50 into the nucleus, and binding to NF-κB DNA regulatory sites. NF-κB activation is known to activate expression of specific target genes encoding proinflammatory and cell cycle regulatory factors, thereby potentially promoting intimal hyperplasia and inflammation seen in filter-implanted vena cava wall

the vein. This new finding results from the low-level phosphorylated IκB-α also present in normal vena cava. Our observation, therefore, is in favor of an explanation that low-level spontaneous phosphorylation of IκB-α in normal vena cava may lead to the continual transfer of a small portion of NF-κB into the nuclei, constituting a basal activity [8]. The basal NF-κB may be necessary for maintaining physiology of a normal vessel.

Mechanical stress is known to activate arterial NF-κB and subsequent intimal hyperplasia [8, 9, 16]. The most studied model is balloon angioplasty-injured arteries [8, 9, 16]. NF-κB activation in arteries following balloon angioplasty is transient, reaching the peak at 6 h after injury and returning to the basal level at 3 days post-injury. This is followed by development of neointimal hyperplasia [16]. Although our study did not address specific kinetics of NF-κB activation after vena cava filter insertion, we noted that significant activation of NF-κB is found in well-developed vena cava intima after 30 days of filter implantation. The NF-κB responses to angioplasty and vena cava filter implantation may all result from mechanical stresses within vessel walls. We postulated that NF-κB activation caused by vena cava filter implantation may be relatively sustained and more pronounced because of sustained filter placement as compared to transient stimulation by balloon inflation. The present work further demonstrates that NF-κB activation is concurrently associated with remarkable phosphorylation of IκB-α in intima caused by the filter placement. This novel finding, therefore, suggests that the filter placement enhances NF-κB DNA-binding activity through phosphorylating intracellular IκB-α and subsequent nuclear translocation of NF-κB subunits p65 and p50.

How mechanical injury affects arterial IκB and NF-κB in vivo remains unknown and is also beyond the scope of the present study with filter-implanted venae cavae. Studies in cell culture have documented that mechanical stretch induces reactive oxygen species-dependent activation of NF-κB leading to expression of numerous genes and cell proliferation [17]. It is, therefore, reasonable to hypothesize that the IκB phosphorylation and NF-κB activation in the injured vena cava may be triggered by inducing reactive

oxygen production. Additionally, cytokines, platelet activation and clot components, secondary to mechanical injury, may also contribute to the NF-κB activity.

Vena cava responses to filter implantation include intima hyperplasia and inflammation. Our study suggests that NF-κB activation may contribute to all the filter-induced vena cava responses since the activity is known to control expression of cell cycle regulators, proinflammatory cytokines and tissue factor. In this regard, one of our previous studies had reported that the filter insertion increased intimal cell expression of cyclin D1, a NF-κB-regulable gene product responsible for cell proliferation [3]. It is, therefore, possible that vena cava filter insertion may induce hyperplastic intima through NF-κB-mediated expression of cyclin D1. This possibility warrants future study.

It has been well documented that agents inhibiting NF-κB DNA binding can effectively minimize hyperplastic response to balloon injury [9]. We, therefore, proposed, as shown in Fig. 5, that therapeutically targeting NF-κB would significantly improve the filter retrieval by minimizing proliferation of intimal cells and inflammation caused by the filter insertion. Further, the present study discloses IκB phosphorylation, nuclear translocation of NF-κB and increase in DNA binding as the sequence of NF-κB activation events. Therefore, molecular or pharmacological agents for inhibiting NF-κB in the vena cava could target against each of these three events. In this regard, dexamethasone and rapamycin, drugs with regulatory activity on IκB/NF-κB, have been used locally to inhibit intimal hyperplasia and restenosis caused by stent placement. It is tempting to speculate that implantation of a filter coated with such drugs might minimize abnormal vena cava response and ease the filter retrieval.

Study Limitations

The limitations of this study were as follows. The present study is on swine vena cava. The swine model has been used extensively by manufactures for the development and testing of vena cava filters [2]. Even so, it is possible that human vena cava and swine vena cava responses to filter implantation are not exactly the same. An additional weakness of this study is relative sample size (four swine). We collected and compared paired NVC and IFVC tissues from the same animal. One important advantage of comparing the paired tissue specimens is to avoid any inter-animal variation. Future research needs to address NF-κB in human intimal tissues associated with the filter struts. Studies with both well-controlled animal tissue experiments and human vascular lesion specimens would collectively yield more convincing information defining therapeutic targets.

Conclusion

The present study demonstrates for the first time that VCF implantation elevates expression and activation of NF-κB p65 and p50 in neointimal cells. We further demonstrate that the activation is at least partly due to phosphorylation of its inhibitor IκB-α. Our data suggest that activation of NF-κB would significantly contribute to development of intimal hyperplasia and inflammation in filter-inserted vena cava walls. NF-κB may be a therapeutic target for inhibiting filter-caused intimal overgrowth and improving filter retrieval.

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Authors' Contribution ZW performed literature search, study design, data interpretation, and writing and critical revision. LK and JK conducted data collection and analysis. DWA, DKN and PSD contributed to critical revision of the manuscript. All provided final approval of the present version for publication.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national and/or institutional guidelines for the case and use of animals were followed.

Consent for Publication For this type of study, consent for publication is not required.

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