Original Article

Novel TNF- α Receptor 1 Antagonist Treatment Attenuates Arterial Inflammation and Intimal Hyperplasia in Mice

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Aim: Tumor necrosis factor receptor 1 (TNFR1) participates importantly in arterial inflammation in genetically altered mice; however it remains undetermined whether a selective TNFR1 antagonist inhibits arterial inflammation and intimal hyperplasia. This study aimed to determine the effect and mechanism of a novel TNFR1 antagonist in the suppression of arterial inflammation.

Methods: We investigated intimal hyperplasia in IL-1 receptor antagonist-deficient mice two weeks after inducing femoral artery injury in an external vascular cuff model. All mice received intraperitoneal injections of TNFR1 antagonist (PEG-R1antTNF) or normal saline twice daily for 14 days.

Results: PEG-R1antTNF treatment yielded no adverse systemic effects, and we observed no significant differences in serum cholesterol or blood pressure in either group; however, selective PEG-R1antTNF treatment significantly reduced intimal hyperplasia ($19,671 \pm 4,274$ vs. $11,440 \pm 3,292$ μm^2 ; $p=0.001$) and the intima/media ratio (1.86 ± 0.43 vs. 1.34 ± 0.36 ; $p=0.029$), compared with saline injection. Immunostaining revealed that PEG-R1antTNF inhibits Nuclear factor- κ B (NF- κ B), suppressing smooth muscle cell (SMC) proliferation and decreasing chemokine and adhesion molecule expression, and thus decreasing intimal hyperplasia and inflammation.

Conclusions: Our data suggest that PEG-R1antTNF suppresses SMC proliferation and inflammation by inhibiting NF- κ B. This study highlights the potential therapeutic benefit of selective TNFR1 antagonist therapy in preventing intimal hyperplasia and arterial inflammation.

J Atheroscler Thromb, 2012; 19:36-46.

Key words; TNF receptor 1 antagonist, Cytokine, Inflammation, Intimal hyperplasia, Smooth muscle cell

Introduction

Tumor necrosis factor- α (TNF- α) possesses important proinflammatory properties that participate crucially in innate and adaptive immunity¹. Due to its numerous effects on different cell types (e.g., macrophages, endothelial cells, and smooth muscle cells

(SMCs)), TNF- α might contribute to arterial inflammation and intimal hyperplasia, including the induction of adhesion molecule expression of cell migration and proliferation^{2,3}.

TNF- α elicits responses through two receptors, TNF receptor 1 (TNFR1) and TNF- α receptor 2 (TNFR2). TNFR1 activates the majority of biological responses⁴. TNFR2 is mainly expressed in cells associated with the immune response, and TNFR2 signaling plays an important role in the biophylactic system⁵. Several previous studies have examined the effect of TNFR1 signaling on vascular physiology using TNFR1-deficient mice. Schreyer *et al.* demonstrated

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Received: April 18, 2011

Accepted for publication: August 9, 2011

an anti-atherogenic effect of TNFR1 signaling⁶). In another study, TNFR1 did not affect atherosclerosis in Apo E-deficient mice⁷). Conversely, Zhang *et al.* demonstrated that TNFR1 expression in the arterial wall contributed substantially to atherosclerosis in an arterial grafting model using TNFR1-deficient mice⁸). Furthermore, they demonstrated that TNF signaling via TNFR2 attenuated neointimal hyperplasia by reducing adhesion molecule expression and endothelial cell apoptosis in an arterial grafting model using TNFR2-deficient mice⁹). Xanthoulea *et al.* showed that atherosclerotic plaques are smaller in LDL receptor-deficient mice carrying TNFR1-deficient bone marrow compared to controls¹⁰). Taken together, these studies suggest that the role of TNF- α , TNFR1 and TNFR2 in vascular inflammation remains incompletely understood. As referred to above, several reports using genetically-altered mice suggested that TNFR1-specific blocking therapy may be the optimal therapy for arterial inflammation; however, no current data show that blocking with a TNFR1 antagonist contributes to the inhibition of arterial inflammation and atherogenesis. We therefore attempted to reveal the roles of these two TNF receptor subtypes in arterial inflammation *in vitro* and *in vivo* using a TNFR1 antagonist.

Recently, two types of TNF blocker, infliximab (chimeric TNF- α monoclonal antibody) and etanercept (soluble TNF receptor), have become available for the treatment of rheumatoid arthritis, and have proven to be efficacious. Previous reports showed that anti-TNF therapy improved endothelial function and decreased cardiovascular events associated with systemic inflammation of rheumatoid arthritis¹¹⁻¹³), but the therapy has its downsides. Inhibition of TNF- α function by anti-TNF therapy increases the chance of infection¹⁴), whereas TNFR1-specific blocking therapy (inhibiting only TNFR1 signal, but not TNFR2 signal) has the potential to inhibit inflammation and offset the side effects of conventional TNF blockers.

More recently, our colleagues produced a novel TNFR1-selective antagonistic mutant TNF- α (R1antTNF) using phage display¹⁵), and also developed PEGylated R1antTNF (PEG-R1antTNF), an agent that further enhances potential anti-inflammatory activity^{16, 17}). The aim of the present study, therefore, was to clarify the effect of this novel TNFR1 antagonist on arterial inflammation and intimal hyperplasia.

Materials and Methods

Novel Tumor Necrosis Factor- α Receptor 1 Antagonist; R1antTNF and PEG-R1antTNF

Our colleagues developed R1antTNF and PEG-R1antTNF for use with a phage-display system. Briefly, they constructed a phage library that displays structural variants of human TNF, in which random amino acid sequences replace the 6 residues (amino acids 84-89) that are likely present in the TNF receptor-binding site from the crystal structure of the LT- α -TNFR1 complex. This phage library consisted of 1×10^7 independent recombinant clones. The phage selection that displays structural TNF variants (Panning) yielded the structural TNF variant R1antTNF with selectively high affinity for TNFR1 and inhibition of TNFR1 signaling. Compared with wild-type TNF- α , R1antTNF has superior affinity for TNFR1, but only one fifty-thousandth of affinity for TNFR2. PEG-R1antTNF is PEGylated R1antTNF, which PEG (polyethylene glycol; average molecular weight 5,000) binds to N-terminal site of R1antTNF, thus improving circulatory retention¹⁵⁻¹⁷).

To compare *in vivo* stability, we injected mice intraperitoneally with PEG-R1antTNF and R1antTNF, and measured serum levels at the indicated time points. In the R1antTNF group, serum concentration almost attained the limit of detection 12 h post-injection. In contrast, the retention time of PEG-R1antTNF in the circulation was considerably longer than R1antTNF¹⁷).

Cell Culture

We purchased human umbilical vein endothelial cells (HUVECs) and human aortic smooth muscle cells (HASMCs) (Kurabo). After culturing HUVECs in HuMedia-EG2 and HASMCs in HuMedia-SG2 (Kurabo) with growth factor, fourth and seventh passage cells, respectively, were used for our experiment. HUVECs and HASMCs were cultured to confluence. After washing the dishes, HUVECs and HASMCs were cultured with 1 ng/mL human recombinant TNF- α (R&D systems) and 1 μ g/mL R1antTNF for 5, 10, 30, 60 and 120min. The cells were lysed with Lysis Buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, 50 mM NaF, 30 mM Na₄O₇P₂, 1 mM Na₃VO₄, protease inhibitor) and used for Western blotting.

SDS-PAGE and Western Blotting

Cell lysate and 2x protein sample buffer for SDS-PAGE (BIO-RAD) were mixed equally, and then 2-mercaptoethanol was added at a final concentration

of 5%. After incubation at 100°C for 10min, these samples were separated by 10-20% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Hoefer). The membrane was incubated with rabbit anti-phospho-nuclear factor-kappa B (pNF- κ B) (Cell Signaling) diluted 1:1000, rabbit anti-phospho-endothelial/epithelial tyrosine kinase (pEtk) (Cell Signaling) diluted 1:1000 and mouse anti-human β -actin (BD Bioscience) diluted 1:30000 in 4% BlockAce (Dainippon Sumitomo Pharma), and then treated with goat anti-rabbit IgG-horseradish peroxidase (Cell Signaling) diluted 1:2000 and goat anti-mouse IgG-horseradish peroxidase (Sigma Aldrich) diluted 1:50000, respectively. Immunodetection was performed with chemiluminescent reagent; ECL-plus (GE Healthcare). The immunoblot was analyzed with an imaging system (LAS3000; Fuji Film), and bands density was estimated using Image J 1.14 (National Institutes of Health).

Reverse Transcription Quantitative PCR *in vitro*

We cultured HUVECs with 1 ng/mL human recombinant TNF α (R&D Systems) and 1 μ g/mL R1antTNF for 24 hours. Total RNA from HUVECs was isolated with TRIzol (Invitrogen). Complementary DNA was obtained using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative mRNA expression was assessed by real-time PCR with Taqman PCR Master Mix (Applied Biosystems) using specific primers for ICAM-1 (Taqman gene expression assay; Applied Biosystems). Samples were run in duplicate on the 7900HT Fast Real-Time OCR system (Applied Biosystems).

Mice and Genotyping

Interleukin-1 receptor antagonist deficiency (IL-1Ra $^{-/-}$) mice were generated in our laboratory by replacing the exons encoding the secreted form with the neo gene, as previously described¹⁸. IL-1Ra $^{-/-}$ mice showed excessive arterial inflammation and intimal hyperplasia after cuff injury¹⁹.

Embryonic stem cells were aggregated with 2 (C57BL/6J $\times$ DBA/2) F1 mice at the 8-cell stage. All 4 isoforms of IL-1Ra were destroyed and mutant mice were a background to C57BL/6J strain mice for 8 generations. To obtain homozygous mutant mice, heterozygous mice were intercrossed with each other. All studies were conducted according to the protocols approved by the National Defense Medical College Board for Studies in Experimental Animals.

Femoral Artery Injury and Treatment

To eliminate gender differences, we used only male mice. Eight-week-old mice were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). We dissected the left femoral artery from its surrounding, as described previously²⁰. Vascular injury was inflicted by placing a non-occlusive polyethylene cuff (length 2.0 mm; internal diameter 0.56 mm; Becton Dickinson) around the femoral artery. Mice received intraperitoneal injections of PEG-R1antTNF (experimental model; 3 μ g twice daily) or normal saline (controls) twice daily for two weeks.

Plasma Lipid Measurement

A blood sample was collected from both groups at 14 days post-injury. Plasma total cholesterol, low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL) cholesterol levels were measured by high performance liquid chromatography (HPLC) at Skylight Biotech Inc. (Akita, Japan) as described previously²¹. Plasma lipoproteins were analyzed by an on-line dual enzymatic method for simultaneous quantification of cholesterol according to the procedure described by Usui *et al.*²².

Arterial Harvest and Morphometric Analysis

After measuring systolic blood pressure, the animals were euthanized by pentobarbital injection and the vascular tree perfused with 0.9% NaCl followed by 4% paraformaldehyde. Following perfusion, the femoral artery was harvested and fixed with 10% neutral-buffered formalin.

We embedded the artery in paraffin and cut 10 sections (each 5- μ m thick) from 10 equally spaced locations. The slides were stained with hematoxylin-eosin and elastica van Gieson, and then examined and photographed using ECLIPS LV100 microscopy (Nikon). The luminal, neointimal, and medial areas were calculated using NIS element (Nikon). To determine the effect of PEG-R1antTNF on vascular remodeling, we defined the vessel area as the area inside external elastic lamina, and calculated the intima area/vessel area ratio.

Immunohistochemistry

Using anti-proliferating cell nuclear antigen (PCNA) antibody (1:100; Dako), α smooth muscle actin (SMA) antibody (Dako), and pNF- κ B antibody (1:50; Santa Cruz Biotechnology, Santa Cruz), respectively, we conducted immunohistochemistry for proliferating, SMCs, and NF- κ B activation on paraffin-embedded sections. Before immunostaining, sections were treated in a microwave oven in 0.1 mol/L citrate

buffer, pH 6.0. Endogenous peroxidase was blocked by incubation with 3% H₂O₂ in methanol for 5 min. Slides were incubated with normal swine serum (Vector Laboratories) for 10 minutes and then with primary antibody overnight at 4°C at the concentrations described above. The sections were incubated with the complementary secondary antibody for 60 min. We visualized the sections using the Envision system (Dako) with DAB as the substrate. Conversely, we conducted immunohistochemistry on frozen sections of monocytes/macrophages, leukocyte adhesion molecules, and chemokines, using anti-CD11b antibody (1:50; BD Bioscience), anti-intracellular adhesion molecule 1 (ICAM-1) antibody (1:50 R&D Systems), and monocyte chemoattractant protein 1 (MCP-1) antibody (1:100; Santa Cruz Biotechnology), respectively. The secondary antibody was the biotinylated antibody (Dako). The sections were visualized with a Vectastain ABC kit (Vector Laboratories), using DAB as the substrate. The nuclei were counterstained with Mayer's hematoxylin solution. Negative control slides were incubated without a primary antibody.

Reverse Transcription Quantitative PCR

Total RNA from the liver, lung, thymus gland and femoral artery tissue was isolated with TRIzol (Invitrogen). Complimentary DNA was obtained using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative mRNA expression was assessed by real-time PCR with Power SYBR Green PCR Master Mix (Applied Biosystems) using primers specific for TNF- α (forward: TCC CAG GTT CTC TTC AAG GGA, reverse: GGT GAG GAG CAC GTA GTC GG), MCP-1 (forward: CCA CTC ACC TGC TAC TCA T, reverse: TGGTGA TCC TCTTGT AGC TCT CC) and GAPDH (forward: AAC TTT GGC ATT GTG GAA GG, reverse: ACA CAT TGG GGG TAG GAA CA). Samples were run in duplicate on the 7900HT Fast Real-Time PCR system (Applied Biosystems).

Statistical Analysis

Results are shown as the mean $\pm$ SD. Differences between groups were analyzed by Student's *t* test. *P* < 0.05 was regarded as significant.

Results

R1antTNF Limited TNF α Induced NF- κ B Activation in Endothelial Cells and Smooth Muscle Cells

Western blotting was used to examine pNF- κ B expression in HUVECs and SMCs to ascertain whether R1antTNF inhibited TNFR1 signaling. pNF- κ B

peaked 30min after TNF- α stimulation and then deteriorated quickly. R1antTNF decreased pNF- κ B expression in both HUVECs and SMCs compared with the control (**Fig. 1A**). Calculation of the pNF- κ B/ β -actin expression ratio 30 min after TNF- α stimulation showed that R1antTNF inhibition of pNF- κ B expression was superior to the control in HUVECs (2.03 ± 0.57 vs. 1.30 ± 0.08 ; *p* = 0.045) and SMCs (1.74 ± 0.16 vs. 1.17 ± 0.26 ; *p* = 0.009) (**Fig. 1B**).

To determine the influence on TNFR2 signaling, we investigated the specific effect of phosphorylated pEtk on TNFR2 signaling. pEtk peaked 5 min after TNF- α stimulation in endothelial cells. We observed no significant differences in pEtk expression between groups (**Fig. 1C**).

Next, we evaluated mRNA levels of ICAM-1 in HUVECs using real-time PCR. Analysis revealed the reduction of mRNA levels of ICAM-1 in HUVECs with both TNF- α and R1antTNF compared with those with TNF- α only (2.11 ± 0.38 vs. 1.54 ± 0.23 ; *p* = 0.042) (**Fig. 2**).

PEG-R1antTNF Treatment Yielded No Adverse in IL-1Ra-/- Mice

We investigated intimal hyperplasia in IL-1Ra-/- mice two weeks after femoral artery injury by an external vascular cuff model. Mice received intraperitoneal injections of PEG-R1antTNF (experimental model) or normal saline (controls) twice daily for two weeks. No adverse systemic effects of PEG-R1antTNF were observed and systolic blood pressure was similar between groups (**Table 1**). Moreover, plasma lipid analysis revealed no statistically significant differences in total cholesterol, LDL cholesterol, and HDL cholesterol between these groups (**Table 2**).

To examine how R1antTNF influences the whole body, we evaluated TNF- α mRNA expression levels in the liver, lung, and thymus gland two weeks after administration using reverse transcription quantitative PCR. We observed a tendency toward inhibited TNF- α mRNA expression in the liver of the PEG-R1antTNF group, but the difference was not statistically significant compared with controls (1.98 ± 1.41 vs. 0.66 ± 0.33 ; *p* = 0.075). Expression levels of TNF- α mRNA in the lung and thymus gland of the PEG-R1antTNF group did not differ significantly from the controls (**Table 3**).

PEG-R1antTNF Inhibited Intimal Hyperplasia in IL-1Ra-/- Arteries Post-Injury

We investigated the effect of PEG-R1antTNF treatment on the femoral arteries of IL-1Ra-/- mice following cuff-induced injury. **Fig. 3** shows representa-

Fig. 1. R1antTNF significantly inhibited the expression of pNF- κ B in HUVECs and SMCs.

(A) Expression levels of pNF- κ B peaked 30 min after stimulation in Western blot analysis. (B) Bar graphs show band density 30 min after stimulation in control and R1antTNF treatment. Data are expressed as the mean $\pm$ SD ($n=4$). * $p<0.05$, ** $p<0.01$. (C) There was no difference in the expression levels of pEtk between R1antTNF and the control group in Western blot analysis.

tive cross sections of femoral arteries harvested 14 days post-injury. Immunostaining for α -SMA showed that intimal hyperplasia consisted of SMCs. PEG-R1antTNF but not saline treatment inhibited intimal hyperplasia (**Fig. 3A**). Morphometric analysis revealed significantly decreased intimal hyperplasia in mice receiving PEG-R1antTNF treatment compared with controls ($19,671 \pm 4,274$ vs. $11,440 \pm 3,292$ μm^2 ;

$p=0.001$) (**Fig. 3B**). PEG-R1antTNF treatment also decreased the intima/media ratio (1.86 ± 0.43 vs. 1.34 ± 0.36 ; $p=0.029$) and the intima/vessel area ratio (0.35 ± 0.06 vs. 0.21 ± 0.11 ; $p=0.011$) compared with saline controls (**Fig. 3C and D**); therefore, our results suggest that PEG-R1antTNF significantly decreases intimal hyperplasia.

Fig. 2. R1antTNF inhibited the expression of ICAM-1 mRNA in HUVECs after TNF- α stimulation. mRNA levels of ICAM-1 were assessed with real-time PCR. Data are expressed as the mean $\pm$ SD ($n=4$). * $p<0.05$.

Inhibition of NF- κ B Activation by PEG-R1antTNF Decreased Expression of Chemokine and Adhesion Molecule

To examine TNF- α and MCP-1 expression in the injured artery 5 days post-surgery, real-time PCR was performed to evaluate mRNA levels of TNF- α and MCP-1. Mice receiving PEG-R1antTNF treatment showed inhibited expression of TNF- α (2.62 ± 1.47 vs. 1.06 ± 0.74 ; $p=0.029$) and MCP-1 (1.34 ± 0.77 vs. 0.50 ± 0.24 ; $p=0.048$) mRNA compared with control mice (**Fig. 4A**).

To determine the effect of PEG-R1antTNF on injured arteries, immunostaining for pNF- κ B was performed at 7 and 14 days post-injury. Fewer pNF- κ B-positive nuclei were observed in the intima of PEG-R1antTNF-treated mice at 7 and 14 days than in controls (**Fig. 4B**). ICAM-1 and MCP-1 expressions were also investigated, and both increased in endothelial cells from controls but not PEG-R1antTNF-treated mice (**Fig. 4C**). Immunostaining also revealed fewer macrophages in the intima of PEG-R1antTNF-treated mice than in controls (**Fig. 4C**).

To determine cell proliferating activity, immunostaining for PCNA was performed 14 days post-injury. PEG-R1antTNF decreased the number of PCNA-positive nuclei compared with controls. Furthermore, the expression of PCNA-positive nuclei considerably accorded with α -SMA-positive cells (**Fig. 5A**). Quantitative analysis also revealed that

Table 1. Measurement of blood pressure and heart rate at 14 days post-injured

	Control	R1antTNF	<i>p</i> value
Blood pressure (mmHg)	106.6 $\pm$ 10.0	110.6 $\pm$ 5.3	0.451
Heart rate (bpm)	654.2 $\pm$ 48.6	666.6 $\pm$ 56.9	0.721

Results are expressed as the mean $\pm$ SD

Table 2. Plasma lipid analysis at 14 days post-injured

	Control	R1antTNF	<i>p</i> value
Total Cholesterol (mg/dL)	73.11 $\pm$ 8.30	68.87 $\pm$ 8.37	0.399
LDL Cholesterol (mg/dL)	21.69 $\pm$ 4.47	18.37 $\pm$ 2.33	0.137
HDL Cholesterol (mg/dL)	46.73 $\pm$ 6.75	44.79 $\pm$ 9.02	0.683

Results are expressed as the mean $\pm$ SD

Table 3. TNF- α mRNA expression at 14 days post-injured

	Control	R1antTNF	<i>p</i> value
Liver	1.98 $\pm$ 1.41	0.66 $\pm$ 0.33	0.075
Lung	1.10 $\pm$ 0.18	1.10 $\pm$ 0.74	0.994
Thymus Gland	0.98 $\pm$ 0.53	1.09 $\pm$ 0.32	0.728

Value is TNF- α mRNA/GAPDH mRNA ratio. Results are expressed as the mean $\pm$ SD

PEG-R1antTNF-treated mice had fewer PCNA-positive nuclei than controls 14 days post-injury (**Fig. 5B**). These data suggest that PEG-R1antTNF exerts a vascular anti-inflammatory effect by inhibiting NF- κ B.

Discussion

We demonstrate here for the first time that a specific TNFR1 antagonist inhibited intimal hyperplasia following arterial inflammation induced by cuff injury in IL-1Ra-/- mice with excessive post-injury inflammation. Previous reports demonstrated that TNFR1 participates in exacerbated intimal hyperplasia in wire-injured artery²³⁾ or arteriovenous grafts⁸⁾ using the mouse carotid artery. Moreover, a study using double-deficient (TNFR1 and LDL receptor) mice showed decreasing sizes of atherosclerotic plaque²⁴⁾. Thus, TNF- α plays an important role, and TNF signaling through TNFR1 participates significantly in the development of intimal hyperplasia and atherosclerosis; however, these findings were observed in genetically altered mice, and the current literature does not show how the TNFR1 antagonist might affect arterial inflammation.

Fig. 3. PEG-R1antTNF significantly inhibited intimal hyperplasia in IL-1 Ra^{-/-} arteries post-injury.

(A) Microscopic appearance of hematoxylin and eosin staining (left), elastica van Gieson staining (middle) and α -SMA staining (right) of femoral artery from control (upper panels) and PEG-R1antTNF (lower panels) groups 14 days post-injury. Scale bar = 50 μm . Bar graphs show intima area (B), intima/media area ratio (C), and intima/vessel area ratio (D). Data are expressed as the mean $\pm$ SD ($n=7$ for each). * $p<0.05$, ** $p<0.01$.

The present study investigated the relationship between TNF signaling and arterial inflammation, revealing for the first time that TNFR1 signaling blocked by a TNFR1 antagonist might affect arterial inflammation and intimal hyperplasia in cuff-injured IL-1Ra^{-/-} mice with severe inflammation around the artery, similar to Takayasu's disease²⁵). Initially, we used wild-type C57BL/6J mice for the cuff injury model, but treatment induced little neointima formation in the control group. The lack of significant dif-

ferences in intima area ($3,222.9 \pm 1,640.3$ vs. $2,383.9 \pm 618.7 \mu\text{m}^2$; $p=0.267$) and the intima area/media area ratio (0.278 ± 0.119 vs. 0.201 ± 0.060 ; $p=0.179$) between the control and R1antTNF treatment group made it difficult to examine whether R1antTNF suppresses arterial inflammation and intimal hyperplasia. Previously, we reported that deficiency of IL-1Ra promotes intimal hyperplasia after femoral artery injury^{18, 26}). We further determined that TNF- α deficiency suppresses aortitis in IL-1Ra^{-/-} mice²⁷). Thus,

Fig. 4. PEG-R1antTNF suppressed TNF- α , adhesion molecule and chemokine, and activation of NF- κ B.

(A) These graphs showed the amount of TNF- α (left; $n=7$) and MCP-1 (right; $n=5$) mRNA expression 5 days post-injury. Data are expressed as the mean $\pm$ SD. * $p<0.05$. The result of immunostaining for pNF- κ B-positive nucleus 7 and 14 days post-injury (B), expression of ICAM-1 (C; left), MCP-1 (C; middle), and CD11b (C; right) 7 days post-injury. Scale bar=50 μ m.

our findings suggest that TNF- α participates importantly in the development of arterial inflammation in IL-1Ra $^{-/-}$ mice. Consequently, we used IL-1Ra $^{-/-}$ mice in the present study because we believe that these mice were suitable for evaluating the effect of TNFR1 blocking therapy in active arterial inflammation induced by cuff injury.

The dose of PEG-R1antTNF in our study was

determined according to a previous report showing PEG-R1antTNF was effective for the suppression of collagen-induced arthritis in mice¹⁷. The PEG-R1antTNF dose was not changed based on body weight, as infliximab and etanercept are also injected at the same dose independently of the body weight of the patients. Taken together, we think that the dose of PEG-R1ant TNF must be relevant in our study.

Fig. 5. PEG-R1antTNF inhibited cell proliferation in injured arteries.

(A) Representative photographs of α -SMA and PCNA staining of injured arteries 14 days post-injury. Scale bar = 50 μ m. (B) Bar graph shows the number of PCNA-positive nuclei in the intima. Data are expressed as the mean $\pm$ SD ($n=6$). ** $p<0.01$.

A previous report showed that TNF- α activates IKK, induces the phosphorylation and ubiquitination of I κ B, and activates NF- κ B²⁸. NF- κ B is activated mainly via TNFR1 signaling, but only poorly via TNFR2-TNF receptor-associated factor 2 signaling²⁹. NF- κ B regulates macrophage migration and the expression of adhesion factor (ICAM-1)³⁰ and chemokine (MCP-1). NF- κ B also regulates both the pro-

liferation and migration of vascular SMCs³¹⁻³⁴. Our results showed that blocking TNFR1 with R1antTNF decreased macrophage accumulation as well as ICAM-1 and MCP-1 expression by inhibiting NF- κ B activation in endothelial cells and SMCs in the injured artery. Thus, TNFR1 signaling participates importantly in the development of intimal hyperplasia following arterial inflammation. Additionally, we show a relationship among inhibited intimal hyperplasia, suppressed NF- κ B activation, adhesion factor and chemokine expression.

Zhang *et al.* examined the effect of TNF signaling through TNFR2 for intimal hyperplasia, demonstrating that TNFR2 signaling inhibits neointimal formation by decreasing adhered cells and endothelial cells apoptosis. They also showed that Etk/Bmx, a non-receptor tyrosine kinase, contributed to these results⁹. A previous study observed the anti-apoptotic effect of TNFR2, which specifically activates Etk/Bmx. TNFR2 also induces the activation, proliferation and migration of endothelial cells. Another study suggested that Etk/Bmx participates importantly in angiogenesis induced by TNF- α ³⁵. Thus, TNFR1-specific blocking therapy might aid the earlier regeneration of endothelial cells and inhibit intimal hyperplasia compared with TNF blocking therapy, which blocks both TNFR1 and TNFR2 signaling. Therefore, we examined whether R1antTNF treatment altered pEtk/Bmx expression in endothelial cells. Our results revealed no significant difference in pEtk/Bmx expression between groups. Similarly, our colleague, who used an index of GM-CSF production by TNF- α in PC60-R2 cells (a mouse-rat fusion hybridoma consisting of human TNFR2-transfected PC60 cells)¹⁵, reported that R1antTNF did not affect bioactivity via TNFR2. These data suggest that R1antTNF does not stimulate endothelial cell regeneration in *in vitro* studies. In the future, we will examine whether R1antTNF treatment exerts an effect on Etk/Bmx activation via TNFR2 *in vivo*.

TNF- α forms a trimeric structure with various bioactivities³⁶. We found that R1antTNF reacted with endogenous TNF- α to form a heterotrimer *in vitro* (unpublished data), possibly affecting the half-life and bioactivity of endogenous TNF- α *in vivo*. Thus, it remains undetermined whether the *in vivo* effects of R1antTNF might result only from inhibited TNFR1 signaling. Determining whether the trimer formation of R1antTNF and endogenous TNF- α might change the effect will require further study.

TNF blocking therapy improves vascular endothelial dysfunction and reduces cardiovascular events associated with inflammation in patients with rheu-

matoid arthritis^{12, 13}); however, there has been much concern over the reactivation of viral infection caused by TNF blockade¹⁴. Shibata *et al.* compared the effects of PEG-R1antTNF and etanercept on antiviral immunity using a recombinant adenovirus vector and showed that PEG-R1antTNF did not reactivate viral infection, unlike etanercept¹⁷. Their results indicated that use of PEG-R1antTNF may reduce side effects such as increased susceptibility to viral infection due to its TNFR1 selectivity. Additionally, transmembrane TNF (tmTNF), the prime activating ligand of TNFR2⁵, was reported to be sufficient to control *Mycobacterium tuberculosis* infection^{37, 38}, indicating the importance of TNF/TNFR2 function in this bacterial infection. Because PEG-R1antTNF does not bind TNFR2, PEG-R1antTNF cannot inhibit the interaction of tmTNF with TNFR2; therefore, we believe that PEG-R1antTNF would also have reduced side effects in the context of bacterial infection.

In conclusion, the present study shows that a selective TNFR1 antagonist, PEG-R1antTNF, suppresses arterial inflammation by inhibiting NF- κ B activation and chemokine expression. Thus, inhibited TNFR1 signaling may provide a new therapeutic target for preventing intimal hyperplasia and inflammation.

Acknowledgements

We thank Mr. Shigehiro Kitada and Ms. Kozue Suzuki (Division of Basic Pathology, National Defense Medical College) for pathological support. We also thank the staff of the Institute of Laboratory Animals, National Defense Medical College for animal care.

Notice of Grant Support

This research was supported in part by National Defense Medical College Grant for Young Scientist (M.K.) and National Defense Medical College grant H14 (K.I.) and H18 (K.I.)

Disclosure

None.

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