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

Manabu Kitagaki1, Kikuo Isoda2, Haruhiko Kamada3, Takayuki Kobayashi4, Shinichi Tsunoda3, Yasuo Tsutsumi3,5, Tomiharu Niida2, Takehiko Kujiraoka2, Norio Ishigami2, Miya Ishihara1, Osamu Matsubara4, Fumitaka Ohsuzu2 and Makoto Kikuchi1

1Medical Engineering, National Defense Medical College, Saitama, Japan
2Internal Medicine I, National Defense Medical College, Saitama, Japan
3Laboratory of Biopharmaceutical Research, National Institute of Biomedical Innovation, Osaka, Japan
4Basic Pathology, National Defense Medical College, Saitama, Japan
5Department of Toxicology and Safety Science, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan

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 ± 4,274 vs. 11,440 ± 3,292 μm²; 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-κB (NF-κB), 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-κB. This study highlights the potential therapeutic benefit of selective TNFR1 antagonist therapy in preventing intimal hyperplasia and arterial inflammation.


Key words; TNF receptor 1 antagonist, Cytokine, Inflammation, Intimal hyperplasia, Smooth muscle cell
an anti-atherogenic effect of TNFR1 signaling. In another study, TNFR1 did not affect atherosclerosis in Apo E-deficient mice. Conversely, Zhang et al. demonstrated that TNFR1 expression in the arterial wall contributed substantially to atherosclerosis in an arterial grafting model using TNFR1-deficient mice. 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 mice. Xanthoulea et al. showed that atherosclerotic plaques are smaller in LDL receptor-deficient mice carrying TNFR1-deficient bone marrow compared to controls. 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 arthritis, but the therapy has its downsides. Inhibition of TNF-α function by anti-TNF therapy increases the chance of infection, 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 display, and also developed PEGylated R1antTNF (PEG-R1antTNF), an agent that further enhances potential anti-inflammatory activity. 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-TNF complex. This phage library consisted of 1 x 10⁹ 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 retention.

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 R1antTNF.

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, 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 μg/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 NaO4-P2, 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-mercaptethanol was added at a final concentration...
of 5%. After incubation at 100°C for 10 min, 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-κB) (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 1:50000 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).

**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 previously. 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 μg 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 previously. 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.

**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 dissected the left femoral artery from its surrounding vascular tree, and then treated with goat anti-phospho-nuclear factor-kappa B (pNF-κB) (Cell Signaling) diluted 1:1000, rabbit anti-smooth muscle α-actin (SMA) antibody (Dako), and pNF-κB 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 GTA GCG), 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 ± SD. Differences between groups were analyzed by Student’s t test. P < 0.05 was regarded as significant.

**Results**

**R1antTNF Limited TNFα Induced NF-κB Activation in Endothelial Cells and Smooth Muscle Cells**

Western blotting was used to examine pNF-κB expression in HUVECs and SMCs to ascertain whether R1antTNF inhibited TNFR1 signaling. pNF-κB peaked 30 min after TNF-α stimulation and then deteriorated quickly. R1antTNF decreased pNF-κB expression in both HUVECs and SMCs compared with the control (Fig. 1A). Calculation of the pNF-κB/β-actin expression ratio 30 min after TNF-α stimulation showed that R1antTNF inhibition of pNF-κB 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-
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 ± 4,274 vs. 11,440 ± 3,292 μm²; 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. 1.** R1antTNF significantly inhibited the expression of pNF-κB in HUVECs and SMCs.

(A) Expression levels of pNF-κB 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±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.
Inhibition of NF-κB 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-κB was performed at 7 and 14 days post-injury. Fewer pNF-κB-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 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-κB.

**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 artery or arteriovenous grafts using the mouse carotid artery. Moreover, a study using double-deficient (TNFR1 and LDL receptor) mice showed decreasing sizes of atherosclerotic plaque. 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.

---

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

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>R1antTNF</th>
<th><em>p</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure (mmHg)</td>
<td>106.6 ± 10.0</td>
<td>110.6 ± 5.3</td>
<td>0.451</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>654.2 ± 48.6</td>
<td>666.6 ± 56.9</td>
<td>0.721</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± SD

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

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>R1antTNF</th>
<th><em>p</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol (mg/dL)</td>
<td>73.11 ± 8.30</td>
<td>68.87 ± 8.37</td>
<td>0.399</td>
</tr>
<tr>
<td>LDL Cholesterol (mg/dL)</td>
<td>21.69 ± 4.47</td>
<td>18.37 ± 2.33</td>
<td>0.137</td>
</tr>
<tr>
<td>HDL Cholesterol (mg/dL)</td>
<td>46.73 ± 6.75</td>
<td>44.79 ± 9.02</td>
<td>0.683</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± SD

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

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>R1antTNF</th>
<th><em>p</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>1.98 ± 1.41</td>
<td>0.66 ± 0.33</td>
<td>0.075</td>
</tr>
<tr>
<td>Lung</td>
<td>1.10 ± 0.18</td>
<td>1.10 ± 0.74</td>
<td>0.994</td>
</tr>
<tr>
<td>Thymus Gland</td>
<td>0.98 ± 0.53</td>
<td>1.09 ± 0.32</td>
<td>0.728</td>
</tr>
</tbody>
</table>

Value is TNF-α mRNA/GAPDH mRNA ratio. Results are expressed as the mean ± SD.
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 disease\(^{25}\). 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 differences in intima area (3,222.9 ± 1,640.3 vs. 2,383.9 ± 618.7 μm\(^2\); \(p=0.267\)) and the intima/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-\(\alpha\) deficiency suppresses aortitis in IL-1Ra-/- mice\(^{27}\). Thus,

**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 \(\alpha\)-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 intimal area (B), intima/media area ratio (C), and intima/vessel area ratio (D). Data are expressed as the mean ± SD (n = 7 for each). *\(p<0.05\), **\(p<0.01\).
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.
A previous report showed that TNF-α activates IKK, induces the phosphorylation and ubiquitination of IκB, and activates NF-κB. NF-κB is activated mainly via TNFR1 signaling, but only poorly via TNFR2-TNF receptor-associated factor 2 signaling. NF-κB regulates macrophage migration and the expression of adhesion factor (ICAM-1) and chemokine (MCP-1). NF-κB also regulates both the proliferation and migration of vascular SMCs. Our results showed that blocking TNFR1 with R1antTNF decreased macrophage accumulation as well as ICAM-1 and MCP-1 expression by inhibiting NF-κB 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-κB 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 Erk/Bmx, a non-receptor tyrosine kinase, contributed to these results. A previous study observed the anti-apoptotic effect of TNF-α, which specifically activates Erk/Bmx. TNFR2 also induces the activation, proliferation and migration of endothelial cells. Another study suggested that Erk/Bmx participates importantly in angiogenesis induced by TNF-α. 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 pErk/Bmx expression in endothelial cells. Our results revealed no significant difference in pErk/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), 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 Erk/Bmx activation via TNFR2 in vivo.

TNF-α forms a trimeric structure with various bioactivities. 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-
TNFR1 Antagonist Reduces Neointima

matoid arthritis; however, there has been much concern over the reactivation of viral infection caused by TNF blockade. 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 etanercept. 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 TNFR2, was reported to be sufficient to control Mycobacterium tuberculosis infection, 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-κB 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.

References


33) Zhou Z, Connell MC, MacEwan DJ: TNFR1-induced NF-kappaB, but not ERK, p38MAPK or JNK activation, mediates TNF-induced ICAM-1 and VCAM-1 expression on endothelial cells. Cell Signal, 2007; 19: 1238-1248


36) Smith RA, Baglioni C: The active form of tumor necrosis factor-alpha induces cell adhesion to human endothelial cells is under dominant control of one TNF receptor type, TNF-R55. J Exp Med, 1993; 177: 1277-1286

37) Olleros ML, Guler R, Corazza N, Vesin D, Eugster HP: Transmembrane TNF is sufficient to initiate cell migration and granuloma formation and provide acute, but not long-term, control of Mycobacterium tuberculosis infection. J Immunol, 2005; 174: 4852-4859