

Journal of Nephrology

The NF- κ B specific inhibitor DHMEQ prevents thrombus formation in a mouse model of antiphospholipid syndrome

Misato Nishimura¹, Tokiko Nii¹, Gulzhan Trimova¹, Shuhei Miura¹, Kazuo Umezawa², Akira Ushiyama³, Tetsuo Kubota^{1,*}

¹Tokyo Medical and Dental University Graduate School of Health Care Sciences, Tokyo, Japan.

²Department of Molecular Target Medicine Screening, Aichi Medical University School of Medicine, Aichi, Japan.

³Department of Environmental Health, National Institute of Public Health, Saitama, Japan.

ARTICLE INFO

Article type:
Original Article

Article history:
Received: 1 November 2012
Accepted: 18 December 2012
Published online: 1 April 2013

Keywords:
Antiphospholipid syndrome
NF-kappa B
Tissue factor
Thrombosis

ABSTRACT

Background: β 2-glycoprotein I (β 2GPI)-dependent antiphospholipid antibodies (aPLs) are considered to play a pivotal pathogenic role in antiphospholipid syndrome (APS) by inducing the expression of tissue factor, inflammatory cytokines, and chemokines, most of which are dependent upon the NF- κ B pathway. Therefore, the NF- κ B is regarded as a promising target for the development of a novel therapeutic strategy. However, progress has been limited owing to the fact that there are no widely-used in vivo models, or highly specific inhibitors.

Objective: This study aimed to test the effects of an NF- κ B-specific inhibitor, DHMEQ, in preventing thrombus formation using an original mouse model of APS.

Materials and Methods: Specificity of a monoclonal aPL WB-6 was examined by ELISA. WB-6 was injected into normal BALB/c mice with or without DHMEQ treatment. A pulse laser was radiated to a cutaneous vein in the window of a dorsal skinfold chamber attached to the mouse and thrombus formation was observed and recorded under a microscope.

Results: WB-6 bound preferentially to the caldiolipin (CL)- β 2GPI complex rather than to CL alone, or β 2GPI alone. WB-6, but not isotype-matched control antibody, induced a prothrombotic state in the mice by inducing tissue factor expression upon circulating monocytes, resulting in thrombus formation at the site of laser-induced endothelial injury. This diathesis was almost completely ameliorated by DHMEQ treatment.

Conclusions: Inhibition of the NF- κ B pathway is a promising strategy for the development of a novel treatment for APS.

Implication for health policy/practice/research/medical education:

APS causes thrombosis in any organs including the kidney, presenting acute or chronic renal failure, hypertension, and proteinuria. Besides antiplatelet and anticoagulant agents which do not completely prevent the recurrence of the thrombotic events in spite of the risk of bleeding tendency, the development of a novel therapeutic strategy using NF- κ B inhibitors appears to be promising.

Please cite this paper as: Nishimura M, Nii T, Trimova G, Miura S, Umezawa K, Ushiyama A, Kubota T. The NF- κ B specific inhibitor DHMEQ prevents thrombus formation in a mouse model of antiphospholipid syndrome. J Nephrology. 2013; 2(2): 114-121. DOI: 10.12860/JNP.2013.19

**Corresponding author:* Dr. Tetsuo Kubota. Tokyo Medical and Dental University Graduate School of Health Care Sciences, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan. Tel/Fax: +81-3-5803-5369, E-mail: tetsuo.kubota.mtec@tmd.ac.jp

1. Background

Antiphospholipid syndrome (APS) is an autoimmune disorder characterized by recurrent arterial or venous thrombosis, unexplained abortion or fetal death, and the persistent presence of circulating antiphospholipid antibodies (aPLs) (1,2). Approximately half of those patients diagnosed with APS do not exhibit any underlying rheumatic or infectious disease (primary APS). The remaining proportion of patients, however, suffer from autoimmune disease (secondary APS), mostly systemic lupus erythematosus (SLE). Any organ can be involved, but venous thrombosis occurs most commonly in deep vein of the legs, while arterial thrombosis most commonly causes brain infarction. Although less common, it has been increasingly recognized that kidney damage can be caused by vascular lesions in the glomeruli, arteriole and interlobular arteries, resulting in acute or chronic renal failure, hypertension, and proteinuria (3-5). For treatment of APS, antiplatelet agents such as aspirin and the anticoagulant agent warfarin are commonly used, but recurrent events cannot be completely prevented, and in addition, anticoagulation is associated with a risk of bleeding complications (6,7).

The molecular mechanisms underlying prothrombotic diathesis in APS has not yet been fully clarified. However, the presence of aPLs that bind to phospholipids indirectly via β 2-glycoprotein I (β 2GPI), especially with lupus anticoagulant (LA) activity, is known to correlate well with thrombotic events (8,9). In *in vitro* assays, anti- β 2GPI antibodies bind to vascular endothelial cells and monocytes, and induce the expression of tissue factor (TF), the major initiator of the clotting cascade, on the surface of such cells (10-13). In fact, in patients with APS, peripheral blood monocytes

express TF (12,14), and higher levels of soluble TF are detected in the plasma than healthy subjects (15). In a previous study, we confirmed that the IgG fraction from patients with SLE who had β 2GPI-dependent aPLs induced the expression of TF on monocytes (16). Furthermore, activated monocytes express inflammatory cytokines such as IL-1 β and TNF α , which in turn stimulate vascular endothelial cells to express chemokines such as CX3CL1 and CCL5. Since CX3CL1 potentially increases the ability of platelets to adhere to collagen (17), and CCL5 potentially induces platelet aggregation (18), these chemokines may also play a pathogenic role in APS. The expression of TF, IL-1 β , TNF α , CX3CL1, and CCL5 is dependent upon the NF- κ B pathway, and can be inhibited by the NF- κ B specific inhibitor (-)-dehydroxymethylepoxyquinomicin (DHMEQ) (16).

Only a very limited number of studies have demonstrated the efficacy of NF- κ B inhibitors on the prevention of thrombosis, mainly because of the lack of suitable animal models. Herein, we established an original mouse model and tested the effects of the NF- κ B specific inhibitor DHMEQ, developed previously in our laboratory, and demonstrated that DHMEQ prevents thrombus formation induced by β 2GPI-dependent aPLs.

2. Objectives

This study aimed to demonstrate the rationality of developing a novel therapeutic strategy for APS using specific inhibitors for the NF- κ B pathway.

3. Materials and Methods

3.1. Animals and cells

The experimental protocols with mice were approved by Tokyo Medical and Dental University Institutional Animal Care and Use Committee, and National Institute of Public Health Animal

Care and Use Committee. Eight week-old male BALB/c mice were purchased from Sankyo Labo Service (Tokyo, Japan). A murine monocytic cell line, RAW264, was provided by the RIKEN BRC (Tsukuba, Japan) through the National Bio-Resource Project of the MEXT, Japan, and cultured in DMEM complete medium containing 10 % fetal calf serum (FCS), 1 % non-essential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin.

3.2. Monoclonal antibodies and NF-κB inhibitor

An IgG monoclonal antibody WB-6 was derived from a lupus-prone male (NZW x BXSB)F1 mouse, which was originally selected by virtue of its binding affinity to cardiolipin (CL)-FCS. The culture supernatant of WB-6 and a control IgG monoclonal antibody TOB-1, which was specific to an irrelevant peptide, was partially purified by salting out with half-saturated ammonium sulfate, dialyzed against Tris-buffered saline (TBS; 25 mM Tris, 140 mM NaCl, pH 7.4), sterilized by passing through a 0.45 µm filter, and stored in aliquots at -20 °C.

The NF-κB inhibitor DHMEQ was synthesized as described previously (19), dissolved in dimethylsulfoxide (DMSO) and kept at -20 °C until use.

3.3. Enzyme linked immunosorbent assay (ELISA)

CL (Sigma-Aldrich, St. Louis, MO, USA) was dissolved to a concentration of 50 µg/ml in ethanol, added to the wells (100 µl/well) of ELISA plates (Immulon 2HB, Thermo Scientific, Rochester, NY, USA) and left for 16 h at 4 °C without a lid to allow drying. Then, 100 µl/well of 10% FCS in TBS, or 10 µg/ml β2-GPI purified from human plasma (Scipac, Kent, UK) in TBS, were added, and incubated for 2 h at room temperature. After washing with TBS, wells were blocked

with 1% gelatin in TBS, washed with TBS, and incubated with WB-6 or 2C10 diluted in 1% gelatin-TBS (100 µl/well) for 1 h at room temperature. After washing with TBS, bound antibody was detected using alkaline phosphatase-labeled anti-mouse IgG antibody and the substrate paranitrophenyl phosphate.

3.4. In vitro induction of TF by aPL

RAW264 cells were plated in 60 mm culture dishes at a concentration of 5×10^5 /dish, kept undisturbed for 24 h in a 37 °C CO₂ incubator, and were stimulated by WB-6 or TOB-1 (500 µg/ml) for 90 min. DHMEQ or vehicle was added 20 min prior to stimulation. After washing with sterile PBS, total RNA was extracted using TRIzol (Life Technologies, Grand Island, NY, USA) and reverse transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). cDNA of mouse TF was amplified with Takara Ex Taq DNA polymerase (Takara, Ohtsu, Japan) using the following primers: forward 5'-CGGGTG-CAGGCATTCCAGAGAA-3', reverse 5'-GTA-AATGGCGGCTCCTCCCAT-3'. The PCR cycling conditions were as follows; initial activation at 95 °C for 1 min, followed by 34 cycles of 96 °C for 10 sec, 66 °C for 20 sec, and 72 °C for 30 sec, followed by extension at 72 °C for 1 min. PCR products were checked by 8% polyacrylamide gel electrophoresis.

3.5. In vivo induction of TF by aPL

One milligram of WB-6, or 200 µl of sterile saline, was injected into the peritoneal cavity of a normal BALB/c mouse with or without the additional injection of 150 µg of DHMEQ, and 90 min later, blood was collected by cardiac puncture under anesthesia. After hemolysis with 0.25 % NaCl, total RNA was extracted using TRIzol,

and reverse transcribed using the SuperScript III First-Strand Synthesis System. Then real-time RT-PCR was performed by Quantitect SYBR Green PCR kit (Qiagen, Hilden, Germany) using the same TF primers as described above. Cycling conditions were as follows; initial activation at 95 °C for 1 min, followed by 40 cycles of 94 °C for 15 sec, 60 °C for 31 sec, and 72 °C for 30 sec. Amplification products were detected as an increased fluorescent SYBR Green signal during amplification cycles using the ABI Prism 7000 Sequence System (Life Technologies) and quantified by reference to standard curves.

3.6. Anticoagulant activity of aPL

One milligram of the monoclonal antibodies or 200 μ l of sterile saline was intraperitoneally injected into a normal BALB/c mouse. Two hours later, blood samples were collected from a tail vein, immediately mixed with 1/10 volume of sodium citrate, and plasma was separated. We employed a chromogenic assay as an alternative to APTT because of limited sample volume. Fifty microliters of the plasma sample diluted x5 with distilled water was added to the wells of 96-well plastic plates placed in a 30 °C incubator. Samples were then mixed with an equal volume of APTT reagent (APTT-SP, Instrumentation Laboratory, Bedford, MA, USA) and incubated for 5 min. Then, chromogenic substrate of FXa (Spectrozyme FXa, American Diagnostica, Stamford, CT, USA) and calcium chloride was added and mixed, and absorbance at 405 nm of the generated free chromophore (pNA) was measured at 10 min intervals. FXa activity was calculated based on the increase in absorbance per 20 min.

3.7. Induction of thrombus formation

Eight-week-old male BALB/c mice were implanted with polyacetal resin dorsal skinfold cham-

bers (DSC) as described previously (20), and provided for experiments 5-7 days after surgery (Fig. 1). One milligram of WB-6 or TOB-1, or 200 μ l of saline was injected into the peritoneal cavity with or without 150 μ g DHMEQ, and 3 h later, Dylight 488-labeled anti-mouse GPIIb β antibody (0.1 μ g/g body weight, Emfret Analytics, Elbelstadt, Germany) was injected via the tail vein. Under anesthesia with isoflurane, the mouse was held on the table of a fluorescence microscope to observe blood flow in small cutaneous vessels in the DSC window. A pulse laser (10 Hz) was radiated to a small vein of 50-80 μ m in diameter for 30 sec using High Energy Solid State Lasers Minilite I (Continuum, Tokyo, Japan), and images were captured every 5 sec for 10 min. Four vessels were irradiated for each mouse, and 3 mice were used for each group. Consequently, 12 experiments were performed for each group. Five researchers reviewed the resultant images in a blind fashion and estimated thrombus formation by the following scores. 1.0: no thrombus formation, 2.0: a small thrombus, which means less than 50 % of the vessel diameter, was formed but disappeared after 10 min, 2.5: a small thrombus was formed and remained after 10 min, 3.0: a large thrombus was formed but disappeared after 10 min, 3.5: a large thrombus was formed and remained after 10 min.



Figure 1. Dorsal skinfold chamber fitted to a BALB/c mouse. The chamber was made from polyacetal resin so that it was lighter and less stressful for mice than the commonly used metal chamber. Several cutaneous blood vessels can be observed in the round transparent window.

4. Results

4.1. Characterization of WB-6

In direct-binding ELISA, WB-6 showed weak reactivity with CL, increased markedly by the addition of FCS (Fig. 2A). WB-6 also showed weak reactivity with FCS alone. To determine whether the enhancing effect of FCS upon the CL binding activity of WB-6 resulted from the presence of β 2-GPI in FCS, which plays a role as a co-factor as described elsewhere (14-16), we tested to use purified β 2-GPI in place of FCS. As expected, WB-6 showed much higher binding affinity to CL- β 2-GPI complexes than CL alone, but did not bind to β 2-GPI alone (Fig. 2B).

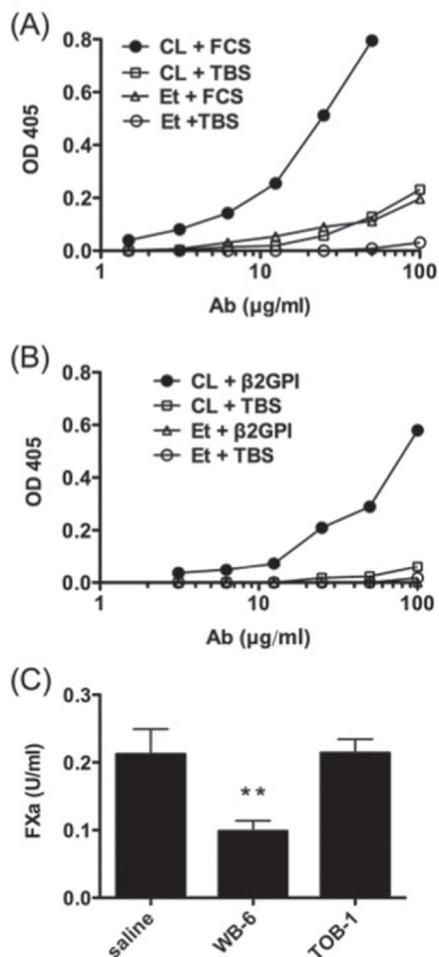


Figure 2. Characterization of WB-6. ELISA plates were first incubated with CL or ethanol (Et), followed by a second incubation with FCS (A), β 2-GPI (B), or diluent (TBS). After blocking with gelatin-TBS, WB-6 was diluted in gelatin-TBS and tested. (C) Lupus anticoagulant-like activity of WB-6. BALB/c mice were intraperitoneally in-

jected with saline, WB-6, or the isotype-matched control antibody TOB-1. Two hours later, plasma APTT activity was measured using a chromogenic substrate for factor X. Bars represent mean \pm SD. ** P < 0.01 by Student's t test.

In patients with APS, the presence of aPL with LA activity has been demonstrated to correlate well with thrombotic events (9). Therefore, we carried out a chromogenic APTT assay to study whether WB-6 has LA-like activity. In the plasma samples from mice injected with WB-6, but not TOB-1, FXa activity was significantly suppressed suggesting that WB-6 exhibits LA-like activity (Fig. 2C).

4.2. Induction of TF by aPL in vitro

To test the prothrombotic activity of WB-6, RAW264 murine monocytic cells were incubated with the antibody for 90 min and the induction of TF mRNA was examined by RT-PCR. TF mRNA was clearly induced by WB-6, but not by TOB-1 (Fig. 3A). This effect of WB-6 was significantly suppressed in the presence of DHMEQ, but not by the vehicle (DMSO).

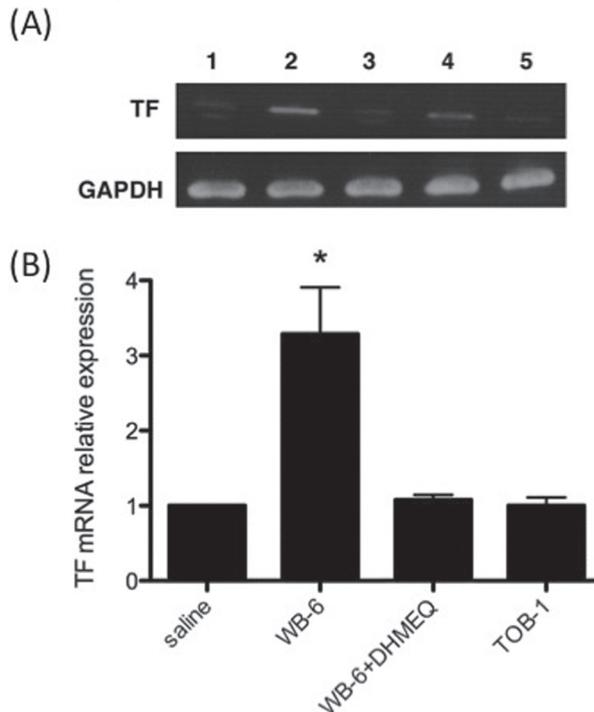


Figure 3. WB-6 induced tissue factor (TF) expression, and associated inhibition by DHMEQ in vitro (A) and in vivo (B). (A) RAW264 cells were stimulated with WB-6 (2-4), isotype-matched control antibody TOB-1 (5), or saline (1)

for 90 min, and TF mRNA expression was detected by RT-PCR. DHMEQ (3) or vehicle (4) was added 20 min before stimulation. (B) BALB/c mice were intraperitoneally injected with saline, WB-6, or TOB-1. 90 min later, TF mRNA expression by circulating white blood cells was estimated by real-time RT-PCR. DHMEQ was injected immediately following WB-6. Bars represent mean \pm SEM. *significantly different from saline ($P < 0.05$) by Mann Whitney test.

4.3. Induction of TF by aPL *in vivo*

To confirm the prothrombotic activity of WB-6 *in vivo*, TF mRNA expression in circulating white blood cells was estimated by real time quantitative RT-PCR in BALB/c mice 90 min after the intraperitoneal injection of antibody. WB-6, but not TOB-1, induced significant TF mRNA expression, and this effect was almost completely suppressed by DHMEQ treatment (Fig. 3B).

4.4. Induction of thrombus formation by aPL and prevention by an NF- κ B inhibitor

As shown above, the β 2GPI-dependent anti-CL antibody WB-6 exhibited TF-inducing activity upon monocytic cells, as well as LA-like activity, suggesting that it is a good candidate antibody to use for the induction of thrombus in a mouse model of APS. As expected, mice injected with WB-6 easily formed a thrombus at the site of endothelial injury caused by pinpoint pulse-laser irradiation. Typically, 1-2 min after radiation, a small thrombus appeared at the site which grew with time and reached maximal size after 5-6 min, thereafter small fragments started to break away resulting in reduction of the thrombus (Fig. 4). In contrast, in the mice injected with TOB-1 or saline, a small thrombus was occasionally formed, but in most cases, a thrombus was not formed. Furthermore, in the mice injected with WB-6, thrombus formation was almost completely suppressed by the simultaneous injection of DHMEQ.

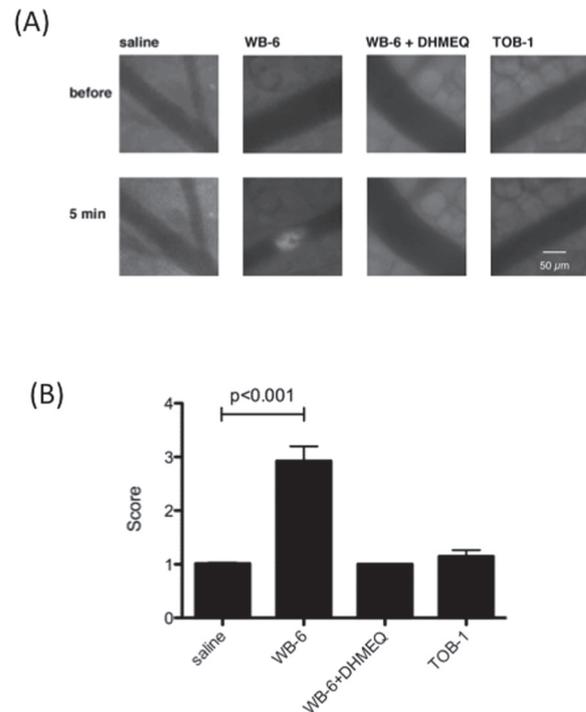


Figure 4. WB-6 induced a thrombophilic state and DHMEQ prevented thrombus formation. BALB/c mice were intraperitoneally injected with saline, WB-6, or isotype-matched control antibody TOB-1. Some mice were injected with DHMEQ immediately following WB-6. Three hours later, a small vein in the skin was irradiated with a pulse laser for 30 sec. (A) Upper panels show vessels before laser irradiation while the lower panels show the same vessels 5 min after irradiation. (B) Thrombus formation score was calculated as described in the Materials and Methods. Bars represent mean \pm SEM of the scores estimated by 5 independent researchers. Data were analyzed by Mann Whitney test.

5. Discussion

In the present study, we have established a novel model for APS by use of a DSC fitted to a BALB/c mouse, a β 2GPI-dependent monoclonal aPL, and pulse-laser irradiation. As the laser can be focused upon a small blood vessel with pinpoint accuracy, we could repeat experiments upon several different vessels in a window of a DSC without exerting significant stress upon the mouse been tested. Using this model, we confirmed that aPL can cause a prothrombotic state, and demonstrated that aPL-induced thrombus

formation is preventable by giving an NF- κ B specific inhibitor.

aPLs are known to induce TF expression on monocytes and vascular endothelial cells via the MAP kinase pathways, notably the p38 MAP kinase and MEK-1/ERK pathways (11,12,21). However, an MEK-1/ERK inhibitor PD98059 suppressed TF expression (12,13) but did not suppress NF- κ B (12), and the critical transcription factor underlying aPL-induced TF expression had not yet been fully determined. An NF- κ B inhibitor MG132 was previously shown to prevent thrombus formation in an in vivo model (22), but this compound was not specific to NF- κ B and the potential involvement of other pathways could not be excluded. On the other hand, the specificity of DHMEQ can be explained by the fact that it binds to a cysteine residue in the CEGRSAGSI motif selectively present in the vicinity of the DNA binding site of RelA, c-Rel and RelB of the NF- κ B family proteins, resulting in inhibition of the canonical and alternative pathway of NF- κ B (23,24). Efficacy of DHMEQ has been proven in various animal models of inflammation (25-27), as well as cancers in which NF- κ B-dependent growth factors drive proliferation of the cells (28-30). Now we can add prevention of aPL-induced thrombus to the indicator list of DHMEQ, and continue to study the pharmacokinetics and adverse effects of this compound, with the ultimate aim of administration to humans.

6. Conclusions

NF- κ B is a highly promising target for the development of a novel therapeutic strategy for APS.

Authors' contributions

TN produced monoclonal antibodies. GT and SM characterized the monoclonal antibodies. MN, TN and AU established an animal model for

APS. KU produced an NF- κ B inhibitor. TK designed the research and prepared the manuscript.

Conflict of interest

The authors declared no competing interests.

Funding/Support

This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology in Japan (#24590681). GT was supported by Asia Pacific League of Associations for Rheumatology Fellowship Grant to come from Kazakhstan.

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