Absence of IFN-γ accelerates thrombus resolution through enhanced MMP-9 and VEGF expression in mice

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Deep vein thrombosis (DVT) is a major cause of pulmonary thromboembolism, a leading cause of death in individuals with DVT. Several lines of evidence indicate proinflammatory cytokines such as TNF-α are involved in thrombus formation and resolution, but the roles of IFN-γ remain unclear. To address this issue, we performed ligation of the inferior vena cava to induce DVT in WT and IFN-γ−/− mice. In WT mice, intrathrombotic IFN-γ levels were elevated progressively as the postligation interval was extended. Thrombus size was substantially smaller at 10 and 14 days in Ifng−/− mice than in WT mice. Intrathrombotic collagen content was remarkably reduced at more than 10 days after the ligation in Ifng−/− mice compared with WT mice. The expression and activity of MMP-9, but not MMP-2, was higher at the late phase in Ifng−/− mice than in WT mice. Moreover, intrathrombotic recanalization was increased in Ifng−/− mice, with enhanced Vegf gene expression, compared with that in WT mice. Activation of the IFN-γ/Stat1 signal pathway suppressed PMA-induced Mmp9 and Vegf gene expression in peritoneal macrophages. Furthermore, administration of anti–IFN-γ mAbs accelerated thrombus resolution in WT mice. Collectively, these findings indicate that IFN-γ can have detrimental roles in thrombus resolution and may be a good molecular target for the acceleration of thrombus resolution in individuals with DVT.

Introduction

Deep vein thrombosis (DVT) is multifactorial and often results from a combination of risk factors such as genetic conditions, obesity, drugs, pregnancy, aging, trauma, and malignancy (1–3). DVT is frequently complicated with severe morbidity, leading sometimes to mortality (4–6). Indeed, the American Heart Association reported that approximately 2,000,000 people annually suffered from DVT and that 200,000 of these died from pulmonary thromboembolism, one of the severe complications arising from DVT, in the United States alone. Thus, in order to minimize the mortality of DVT-related pulmonary thromboembolism, accurate diagnosis and prompt therapy for DVT are necessary (5). Standard treatments for DVT aim to prevent death from pulmonary embolism, propagation of the thrombus, and recurrence of venous thromboembolism. At present, anticoagulation with either unfractionated or low–molecular weight heparin, or warfarin is used to inhibit thrombus extension, to allow natural resolution, and to slowly reduce and organize the existing thrombus (4). However, anticoagulation cannot accelerate natural thrombus resolution and reciprocally increases the risk of bleeding. Moreover, any treatment modalities have not been developed to directly lyse thrombus until the present.

The pathogenesis of venous thrombosis involves 3 factors, referred to as Virchow’s triad; endothelial damages, stasis or turbulence of blood flow, and blood hypercoagulability. Damage to the vessel wall prevents the endothelium from inhibiting coagulation and initiating local fibrinolysis. Venous stasis inhibits the clearance and dilution of activated coagulation factors. Finally, congenital or acquired thrombophila promotes coagulation. Several lines of evidence indicate the involvement of proinflammatory cytokines and chemokines in the thrombus formation and resolution (7–10). Indeed, TNF-α and IL-1β can potently activate endothelial cells, increase the expression of adhesion molecules by endothelial cells, and promote thrombosis (11–15). Moreover, TNF-α and IL-1β can induce the expression of chemokines, which can activate leukocytes to accelerate thrombus resolution and intrathrombotic neovascularization (16–18).

IFN-γ exerts pleiotropic effects on NK cells and macrophages, including enhancement of their antiviral and bactericidal activities and upregulation of MHC class II expression on macrophages (19). Moreover, accumulating evidence implicates IFN-γ as a major mediator in various types of disease models (20–25). Furthermore, we previously determined that the IFN-γ/Stat1 signal pathway played an important role in skin wound healing by regulating VEGF expression and the TGF-β signaling pathway (23). The thrombus resolution process resembles wound healing, with the injury nidus being the clot, followed by leukocyte recruitment and eventually matrix accumulation. Indeed, we immunohistochemically detected IFN-γ protein in deep vein thrombi taken from autopsy cases (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI40782DS1). These observations prompted us to explore the pathophysiological roles of IFN-γ in the process of thrombus formation and resolution in a stasis-induced thrombosis model by the use of IFN-γ−/− mice.

We demonstrated that thrombus resolution was accelerated in Ifng−/− mice compared with WT mice without any differences in thrombosis formation. Moreover, the expression of an enzyme with collagenolytic activity, MMP-9, was enhanced at mRNA and enzymatic activity
levels in Ifng−/− mice compared with WT mice. Furthermore, intrathrombotic vascular channels were increased with enhanced VEGF expression in Ifng−/− mice, compared with WT mice. These observations imply that IFN-γ plays a detrimental role in thrombus resolution by suppressing MMP-9 and VEGF expression.

Results

Intrathrombotic IFN-γ expression after the inferior vena cava ligation (IVC). We examined intrathrombotic Ifng gene expression in WT mice at 3, 5, 10, and 14 days after the IVC ligation. IFN-γ mRNA was consistently detected in thrombi and its expression was elevated progressively as thrombi aged (Figure 1A). Likewise, IFN-γ proteins were detected in thrombi and increased progressively after the IVC ligation (Figure 1B). Moreover, double-color immunofluorescence analyses detected IFN-γ mainly in F4/80-positive macrophages (Figure 1C). These observations implied that IFN-γ was produced in thrombi mainly by infiltrating macrophages.

Enhanced thrombus resolution in the absence of IFN-γ. In order to explore the pathophysiological roles of IFN-γ in the development of stasis-induced venous thrombus, we compared thrombus formation between WT and Ifng−/− mice. In WT mice, venous thrombi developed progressively until 5 days but remained similar in size until 10 days after the IVC ligation, decreasing thereafter (Figure 2A). In Ifng−/− mice, thrombi started to reduce in size 10 days after the IVC ligation (Figure 2, A and B) and were smaller than those in WT mice 14 days after the IVC ligation. Because intrathrombotic collagen contents can reflect thrombus mass (18), we determined intrathrombotic collagen contents by the use of histopathological and biochemical methods. In Ifng−/− mice, intrathrombotic collagen area was remarkably reduced compared with that in WT mice (Figure 2, C and D, and Supplemental Figure 2). Similarly, intrathrombotic contents of both collagen and hydroxyproline, a major component of collagen, were significantly diminished in Ifng−/− mice later than 10 days after the IVC ligation, compared with WT mice (Figure 2, E and F). These observations implied that the absence of IFN-γ could accelerate the resolution of stasis-induced venous thrombi.

The absence of IFN-γ had no effects on intrathrombotic leukocyte recruitment and the expression of chemokines, tPA, uPA, and PAI. The potential contribution of intrathrombotic leukocytes to the resolution of venous thrombi (12–18) prompted us to investigate the effects of IFN-γ deficiency on intrathrombotic leukocyte recruitment. Myeloperoxidase (MPO)-positive neutrophils infiltrated thrombi in WT and Ifng−/− mice to similar extents, reaching a maximal level at 1 day after the IVC ligation and decreasing gradually thereafter (Figure 3, A and C). Likewise, F4/80-positive macrophages infiltrated thrombi in WT and Ifng−/− mice to similar extents, reaching a maximal level at 5 days after the IVC ligation and decreasing gradually thereafter (Figure 3, B and D). Similar tendencies were also observed on the infiltration of CD11b-positive cells (data not shown). In line with this, we further detected the gene expression of several chemokines including CXCL1/KC, CXCL2/MIP-2, CCL2/MCP-1, and CCL3/MIP-1α to similar extents in WT and Ifng−/− mice (Supplemental Figure 3). Because the plasmid system is also essential for cell migration and subsequent thrombus resolution (26–28), we examined the intrathrombotic gene expression of tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), and plasminogen activator inhibitor-1 (PAI-1). We failed to detect any significant differences in the expression of these genes between WT and Ifng−/− mice at any time point after IVC ligation (Figure 4). Collectively, the absence of IFN-γ had little, if any, effects on intrathrombotic expression of chemokines, tPA, uPA, and PAI-1, and subsequent leukocyte recruitment.

Enhanced enzymatic activity of MMP-9 but not MMP-2 in Ifng−/− mice. MMP-2 and MMP-9 have crucial roles in collagenolysis during thrombus resolution (18). Thus, we examined the intrathrombotic gene expression and the enzymatic activity of MMP-2 and MMP-9. MMP-2 mRNA and enzymatic activities were detected consistently in thrombi at similar levels in WT and Ifng−/− mice (Figure 5, A, C, and D). MMP9 gene expression was detected in thrombi of WT mice, reaching the maximal level at 5 days after the IVC ligation and declining thereafter (Figure 5B). In Ifng−/− mice, MMP-9 mRNA was detected, to a similar extent, at 5 days, but its levels remained similar until 14 days after the IVC ligation (Figure 5B). Likewise, the enzymatic activities of MMP-9 remained at similar levels in Ifng−/− mice until 14 days after the IVC ligation, whereas its activities decreased in WT mice at 10 days after the ligation.
(Figure 5, C and E). Consistently, later than 10 days after the IVC ligation, intrathrombotic MMP-9–positive cell numbers were higher in Ifng−/− mice than in WT mice (Figure 6, A and B). Moreover, a double-color immunofluorescence analysis demonstrated that F4/80-positive macrophages were a main cellular source of MMP-9 (Figure 6C). Collectively, these observations would imply that IFN-γ might play a detrimental role in thrombus resolution by suppressing the expression of MMP-9 but not MMP-2 in macrophages present in the thrombus.

Enhanced intrathrombotic recanalization in Ifng−/− mice. Intrathrombotic recanalization was presumed to be essential for thrombus resolution (29–31). The number of intrathrombotic vWF-positive channels was significantly increased at 10 and 14 days in Ifng−/− mice compared with WT mice (Figure 7, A and B). Moreover, we examined blood flow within and around the thrombosed IVC by the use of laser Doppler imaging (18). At 5 days after IVC ligation, there were no significant differences in the blood flow of thrombosed IVC between WT and Ifng−/− mice. However, at 10 and 14 days after IVC ligation, the blood flow was recovered in Ifng−/− to larger extents than in WT mice (Figure 7C). Furthermore, intrathrombotic gene expression of VEGF, a potent angiogenic factor, was significantly enhanced in Ifng−/− mice compared with WT mice (Figure 7D). The number of intrathrombotic vWF-positive channels was significantly increased at 10 and 14 days in Ifng−/− mice compared with WT mice (Figure 7, A and B). Moreover, we examined blood flow within and around the thrombosed IVC by the use of laser Doppler imaging (18). At 5 days after IVC ligation, there were no significant differences in the blood flow of thrombosed IVC between WT and Ifng−/− mice. However, at 10 and 14 days after IVC ligation, the blood flow was recovered in Ifng−/− to larger extents than in WT mice (Figure 7C). Furthermore, intrathrombotic gene expression of VEGF, a potent angiogenic factor, was significantly enhanced in Ifng−/− mice compared with WT mice (Figure 7D).
A double-color immunofluorescence analysis demonstrated that F4/80-positive macrophages were a main cellular source of VEGF (Figure 7E). These observations implied that the lack of IFN-γ might increase intrathrombotic recanalization at least partly through enhanced VEGF gene expression in macrophages.

Effects of an MMP inhibitor on thrombus resolution. There are controversies on the roles of MMP-9 in thrombus formation and resolution (18, 32). Hence, in order to define precisely the roles of enhanced MMP expression in this process, we examined the effects of an MMP inhibitor on thrombus resolution. The administration of an MMP-2/9 inhibitor impaired thrombus resolution, as evidenced by thrombus mass and intrathrombotic collagen contents, and thrombosed blood flow (Figure 8, A to C), whereas it had no effects on intrathrombotic VEGF gene expression (Figure 8D). These observations implied that MMPs have important roles in collagenolysis and angiogenesis during thrombus resolution, with few effects on VEGF expression.

IFN-γ/Stat1 signal pathway negatively regulated the gene expression of MMP-9 and VEGF in macrophages. Our in vivo observations suggest that IFN-γ could suppress the gene expression of MMP-9 and VEGF in intrathrombotic macrophages. To validate this hypothesis, we examined the effects of IFN-γ on the gene expression of MMP-9 and VEGF in WT mouse-derived peritoneal macrophages. PMA significantly enhanced Mmp9 and Vegf gene expression in macrophages (Figure 9, A and B). IFN-γ significantly suppressed PMA-induced gene expression of MMP-9 and VEGF and increase in Stat1 phosphorylation (Figure 9, A–D). Reciprocally, these IFN-γ effects were abrogated by AG490, a JAK2 inhibitor (Figure 9, A to D). These observations strongly implied that the IFN-γ/Stat1 signal pathway negatively regulated Mmp9 and Vegf gene expression in macrophages.

Effects of anti–IFN-γ mAbs on thrombus resolution. We finally examined the therapeutic effects of anti–IFN-γ mAbs on the resolution of stasis-induced venous thrombi. When WT mice received anti–IFN-γ mAbs or control IgG at 4, 8, and 12 days after the IVC ligation, thrombus mass and intrathrombotic collagen contents were smaller in anti–IFN-γ mAb–treated WT mice than control IgG-treated WT ones (Figure 10, A and B). Consistently, blood flow was larger in anti–IFN-γ mAb–treated WT mice than in control IgG-treated WT ones (Figure 10C). Concomitantly, the intrathrombotic gene expression of MMP-9 and VEGF was significantly higher in the anti–IFN-γ mAb–treated group than in the control IgG-treated group (Figure 10, D and E). Moreover, the administration of anti–IFN-γ mAbs did not cause any abnormalities in coagulation func-

Figure 4
Intrathrombotic gene expression of tPA, uPA, and PAI-1 determined by real-time RT-PCR. All values represent the mean ± SEM (n = 6 animals).

Figure 5
Intrathrombotic MMP-2 and MMP-9 expression. (A and B) Intrathrombotic gene expression for MMP-2 (A) and MMP-9 (B) in WT and Ifng–/– mice was determined by real-time RT-PCR as described in Methods. All values represent the mean ± SEM (n = 6). **P < 0.01, Ifng–/– versus WT. (C–E) Intrathrombotic MMP-2 and MMP-9 activities were measured by gelatin gel zymography as described in Methods. Representative gel images from 6 independent experiments are shown in C. Gel images were semiquantitatively analyzed; analysis is shown in D and E. All values represent the mean ± SEM (n = 6). **P < 0.01, Ifng–/– versus WT.
tion as revealed by prothrombin time (PT) (control vs. anti–IFN-γ mAbs: 10.8 ± 0.53 s vs. 11.1 ± 0.25 s) and activated partial thromboplastin time (APTT) (control vs. anti–IFN-γ mAbs: 31.3 ± 1.2 s vs. 30.1 ± 0.8 s). These observations demonstrated that IFN-γ blockade was therapeutically effective in accelerating the thrombus resolution without any adverse effects on coagulation function.

**Discussion**

IFN-γ is produced by Th1 lymphocytes, NK cells, and macrophages (19). Moreover, under several pathological conditions, neutrophils also can produce IFN-γ (20, 22). We proved that macrophage-derived IFN-γ could negatively regulate skin wound healing by dampening the expression of VEGF and TGF-β (23). The resemblance between wound healing and thrombus resolution prompted us to investigate IFN-γ expression in thrombus caused by stasis. Indeed, IFN-γ protein was immunohistochemically detected in venous thrombi obtained from human autopsy cases (Supplemental Figure 1). In the present study, IFN-γ was expressed mainly by infiltrating macrophages and its expression was progressively enhanced until 14 days after the initiation of stasis, suggesting that IFN-γ might be involved in thrombus resolution.

Henke and colleagues previously demonstrated that Ifng−/− mice showed similar rates of thrombus resolution in the early phase compared with WT mice, but they did not examine the later phase (18), when intrathrombotic IFN-γ contents were still enhanced until 14 days after the initiation of stasis. Although we failed to detect any significant differences in the thrombus mass between WT and Ifng−/− mice until 5 days after the initiation of stasis, thrombus mass was significantly reduced in Ifng−/− mice compared with WT mice later than 10 days after the initiation of stasis. These observations implied that locally produced IFN-γ might decelerate thrombus resolution in the later phase.

Rats developed significantly larger thrombi when they were rendered neutropenic by the treatment with antipolymorphonuclear leukocyte serum (33). Intrathrombotic neutrophil recruitment was decreased and subsequent thrombus resolution was diminished by genetic disruption of CXCR2, a receptor for CXC chemokines such as CXCL1/KC and CXCL2/MIP-2 with potent chemotactic activities for neutrophils (16). Supporting the notion that macrophage/monocyte recruitment has an important role in thrombus resolution, Ali and colleagues demonstrated that intrathrombus injection of peritoneal macrophages reduced the thrombus size (34). Moreover, the administration of exogenous MCP-1/CCL2, a potent chemoattractant for macrophage, promoted thrombus resolution (17). Conversely, thrombus resolution was impaired with a concomitant reduction in intrathrombotic macrophage recruitment in mice lacking CCR2, a single and specific receptor for CCL2 (18). Thus, these observations indicate that resolution of venous thrombus is regulated by chemokines and subsequent chemokine-mediated neutrophil and/or macrophage recruitment into the thrombi (6–10). In contrast, the absence of IFN-γ failed to affect the intrathrombotic gene expression of CXC and CC chemokines and subsequent leukocyte recruitment. Thus, accelerated thrombus resolution in Ifng−/− mice was not directly ascribed to changes in leukocyte recruitment and/or chemokine expression.

Plasmin is formed from plasminogen by the action of tPA and uPA (26–28). This pathway is presumed mainly to regulate fibrin deposition in vascular trees and to activate pericellular fibrinolysis, which is required for cell migration in tissues. Singh and colleagues (35) demonstrated that the absence of uPA impaired thrombus resolution, indicating the essential involvement of plasmin system in thrombus resolution. Evidence is accumulating to indicate that IFN-γ regulated the gene expression of plasminogen activators and their inhibitor (36–39). However, in the present study, there was no significant difference in the intrathrombotic gene expression of uPA, tPA, and PAI-1 between WT and Ifng−/− mice. Thus, it is unlikely that the absence of IFN-γ could accelerate thrombus resolution by affecting uPA, tPA, and PAI-1 expression.

Collagenolysis is indispensable for thrombus resolution (10). Reduced intrathrombotic collagen contents would indicate enhanced collagenolysis in Ifng−/− mice compared with WT mice. Among several enzymes with collagenolysis activity, MMP-2 and MMP-9 are presumed to have an important role in collagen turnover during thrombus resolution (18). IFN-γ deficiency increased intrathrombotic MMP-9 but not MMP-2 mRNA expression, particularly at the later phase of thrombus resolution. Moreover, MMP-9 activities were higher in Ifng−/− mice than WT mice. Transient over-

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**Figure 6**

Detection of intrathrombotic MMP-9-positive cells (A) Immunohistochemical analysis was performed using anti–MMP-9 pAbs in venous thrombus samples from WT and Ifng−/− mice. Representative results from 6 independent experiments are shown here. Original magnification, ×400. (B) The numbers of MMP-9-positive cells were determined as described in Methods and are shown in B. All values represent the mean ± SEM (n = 6 animals). *P < 0.05; **P < 0.01. Ifng−/− versus WT. (C) A double-color immunofluorescence analysis of MMP-9-positive cells in the thrombi. The samples were immunostained with the combination of anti-F4/80 mAbs and anti-MMP-9 pAbs, as described in Methods. Representative results from 6 independent experiments are shown here (day 10). Original magnification, ×400. Blue, nuclear staining by DAPI.
expression of MMP-9 alone could promote intravascular thrombus formation induced by arterial balloon injury, whereas the combination of MMP-9 and tissue inhibitor of metalloproteinase 1 (TIMP-1) could prevent it (32). TIMP-1 expression was enhanced, to a similar extent, in thrombus formation sites of both strains in this model (our unpublished observations), and consequently, enhanced MMP-9 expression in the early phase may have few effects on thrombus formation in the presence of TIMP-1. Alternatively, discrepant observations may arise from the use of different thrombosis models. Nevertheless, retardation of thrombus resolution by an MMP-2/9 inhibitor suggested the potential roles of MMP-9 in thrombus resolution in the late phase when MMP-9 but not MMP-2 expression was higher in Ifng−/− than WT mice, although the contribution of MMP-2 cannot be completely excluded.

MMP-9 was expressed predominantly by infiltrating macrophages. Because macrophages are a major target cell of IFN-γ, genetic disruption of IFN-γ could modulate MMP-9 expression by macrophages, similarly reported on several other proinflammatory cytokines, which can regulate Mmp9 gene expression (40). Ma and colleagues revealed that the IFN-γ/Stat1 signal pathway suppressed Mmp9 gene expression in human astroglia and HeLa cell lines (41, 42). In line with this, IFN-γ suppressed PMA-induced Mmp9 gene expression together with depression of Stat1 activation in murine peritoneal macrophages, while AG490, a JAK2 inhibitor, abrogated these effects. Thus, IFN-γ would prevent macrophages from transcribing the Mmp9 gene in an autocrine and/or paracrine manner, and IFN-γ deficiency would cancel the suppression, thereby resulting in enhanced MMP-9 expression and subsequent acceleration of collagen degradation in thrombus.

Neovascularization is another key event in the process of thrombus resolution (29–31), as evidenced by retarded thrombus resolution with impaired intrathrombotic neovascularization (16, 18). Several in vitro studies demonstrated that IFN-γ had direct angiogenic effects, as evidenced by the inhibition of capillary growth and development (43, 44). In support, we previously observed that Ifng−/− mice exhibited enhanced angiogenesis during skin wound healing (23). Likewise, intrathrombotic neovascularization was augmented in Ifng−/− mice compared with WT mice. Moreover, MMP-9 also exhibited its proangiogenic activity by mediating basement membrane remodeling for endothelialization of tissue matrix (45, 46), and the inhibition of MMP-9 impaired neovascularization in several conditions such as cardiac ischemia (47). Consistently, when WT mice received an MMP-2/9 inhibitor, intrathrombotic recanalization was reduced with no effects on intrathrombotic Vegf gene expression, thereby resulting in impaired thrombus resolution. Thus, enhanced MMP-9 expression could promote intrathrombotic recanalization in Ifng−/− mice independently of VEGF.

Accumulating evidence indicates that angiogenic growth factors such as VEGF and bFGF are crucial for intrathrombotic neovascularization (48–50). In the present study, the absence or immunoneutralization of IFN-γ augmented Vegf gene expression.
target for the development of therapies that accelerate thrombus resolution in patients with DVT.

Methods

Reagents and Abs. Recombinant mouse IFN-γ and MMP-2/9 inhibitor were purchased from PeproTech and Calbiochem, respectively. PMA and AG490 (a JAK2 inhibitor), were obtained from Sigma-Aldrich. The following mAbs and polyclonal Abs (pAbs) were used for immunohistochemical and double-color immunofluorescence analyses: rat anti-mouse F4/80 mAbs (Dainippon Pharmaceutical Co.), rabbit anti-vWF pAbs (GeneTex), goat anti–IFN-γ pAbs, goat anti-MMP-9 pAbs, goat anti-VEGF pAbs (Santa Cruz Biotechnology Inc.), rabbit anti-phosphorylated–Stat1 (anti–p-Stat1) pAbs (Applied Biological Materials Inc.), rabbit anti-myeloperoxidase (anti-MPO) pAbs (Neomarkers), cyanine dye 3–conjugated (Cy3-conjugated) donkey anti-rat IgG pAbs, and FITC-conjugated donkey anti-rat IgG or goat IgG pAbs (Jackson Immunoresearch Laboratories). For immunoneu-ralization of endogenous IFN-γ, rat anti-murine IFN-γ mAbs (MAB485, clone: 37895) were purchased from R&D Systems.

Mice. Pathogen-free 8- to 10-week-old male BALB/c mice were obtained from SLC and designated as WT mice in this study. Age- and sex-matched Ifng−/− mice, backcrossed to BALB/c mice for at least 8 generations, were used in the following experiments (20). All mice were housed individually in cages under specific pathogen–free conditions during the experiments. All animal experiments were approved by the Committee on Animal Care and Use at Wakayama Medical University.

Statin-induced DVT model. Intravenous thrombus formation was induced as described previously (18). Briefly, mice were deeply anesthetized by i.p. injection of pentobarbital (50 mg/kg body weight). After a 2-cm incision was made along the abdominal midline, the IVC was ligated with a 3-0 silk suture. In another series of experiments, WT mice were i.p. given anti–IFN-γ mAbs, isotype-matched IgG (50 μg/mouse), or MMP-2/9

Figure 8

The effects of an MMP inhibitor on thrombus resolution. WT mice were i.p. administered with an MMP inhibitor as described in Methods. At 14 days after the IVC ligation, thrombus weight (A) and intrathrombotic collagen contents (B), thrombosed blood flow (C), and Vegf gene expression (D) were measured and shown here. All values represent the mean ± SEM (n = 6 animals). *P < 0.05, versus control.
inhibitor (100 μg/mouse) at 4, 8, and 12 days after the IVC ligation. At the indicated time intervals after the IVC ligation, mice were euthanized by an overdose of diethyl ether and intravascular thrombi were harvested and their weights were measured. We used 6 mice per group and repeated all animal experiments at least 3 times. Because we obtained similar results at each experiment, we demonstrated the representative results from the repeated experiments.

Measurement of PT and APTT. Blood samples were taken with 3.8% citrate solution and centrifuged to obtain plasma samples. PT and APTT of citrate PBS (pH 7.2); paraffin-embedded sections (4-μm thick) were made. The sections were stained with H&E or Masson trichrome solution.

Histopathological analyses. At the indicated time intervals after the IVC ligation, thrombi were harvested and fixed in 4% formaldehyde buffered with PBS (pH 7.2); paraffin-embedded sections (4-μm thick) were made. The sections were stained with H&E or Masson trichrome solution.

Immunohistochemical analyses. Deparaffinized sections were immersed in 0.3% H₂O₂ in methanol for 30 minutes to eliminate endogenous peroxidase activity. The sections were further incubated with PBS containing 1% normal serum derived from the same species as the origin of the second antibody and 1% BSA to reduce nonspecific reactions. The sections were incubated with fluorochrome-conjugated secondary Abs (15 μg/ml) at room temperature for 30 minutes, the sections were observed under fluorescence microscopy. In some experiments, nuclei were stained using DAPI (Roche Diagnostics) according to the manufacturer's instructions.

Measurements of leukocytes and neovessels. Intra- and intrathrombotic leukocytes and vascular density were semiquantitatively evaluated. Briefly, after MPO-positive neutrophils, F4/80-positive macrophages or MMP-9–positive cells were enumerated in 5 high-power fields (×1,000) within the thrombus, total numbers in the 5 fields were combined. Intra- and intrathrombotic vWF-positive neovessels with tube-like formation were counted in 5 high-power fields (×1,000), as described previously (18). All measurements were performed by an examiner without a prior knowledge of the experimental procedures.

**Table 1** Sequences of the primers used for real-time RT-PCR

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F, forward primer; R, reverse primer.

**Figure 10** The effects of anti–IFN-γ mAb administration on thrombus resolution. WT mice were i.p. administered anti–IFN-γ mAbs or isotype-matched IgG as described in Methods. At 14 days after the IVC ligation, thrombosis weight (A) and intrathrombotic collagen contents (B), thrombosed blood flow (C), and the gene expression of MMP-9 (D) and VEGF (E) were measured and shown here. All values represent the mean ± SEM (n = 6 animals). *P < 0.05; **P < 0.01, anti–IFN-γ mAbs versus control IgG.

A double-color immunofluorescence analysis. Deparaffinized sections or peritoneal macrophages were incubated with PBS containing 1% normal donkey serum and 1% BSA to reduce nonspecific reactions as previously described (23). Thereafter, the sections were further incubated in the combination of anti-F4/80 and anti–IFN-γ, anti-F4/80 and anti–MMP-9, anti-F4/80 and anti-p-Stat1, or anti-F4/80 and anti-VEGF. All Abs were used at a concentration of 1 μg/ml. After the incubation with fluorochrome-conjugated secondary Abs (15 μg/ml) at room temperature for 30 minutes, the sections were observed under fluorescence microscopy. In some experiments, nuclei were stained using DAPI (Roche Diagnostics) according to the manufacturer's instructions.

IVC blood flow measurement by a laser Doppler. Microvascular IVC blood flow was assessed by laser Doppler imaging (OMEGA-FLO FLO-C1 BV, OMEGAWAVE) as described previously (18). After laparotomy, at pre-IVC ligation, immediate postligation, and at harvest, blood flow through the exposed IVC region of interest was assessed; a 5-second scan with 30-second interval over 4 scanning cycles was performed. Depth was constant and was adjusted for each mouse to ensure the best estimation of the midcoronal IVC section. These scans were saved, and accompanying image software was used to estimate the mean color flow by using a standardized area of analysis. The intensities were reported as percentage of baseline blood flows, specific to each animal to ensure consistency.

Measurement of intrathrombotic hydroxyproline contents. At the indicated time intervals after IVC ligation, venous thrombi were removed and dried for 16 hours at 120°C. Hydroxyproline (HP), a major constituent of collagen, was measured and shown here. All values represent the mean ± SEM (n = 6 animals). *P < 0.05; **P < 0.01, anti–IFN-γ mAbs versus control IgG.
Collagen assay. Thrombus collagen content was estimated by a commercially available kit according to the manufacturer’s instructions (Biclor), as described (18). Collagen amount was then corrected to thrombus weight for each sample (μg/ml/mg of thrombus).

Extraction of total RNAs and real-time RT-PCR. Real-time RT-PCR was performed as described previously (52). Briefly, total RNA was extracted from tissue samples (100 μg) using ISOGEN (Nippon Gene) according to the manufacturer's instructions, and 5 μg of total RNA was reverse transcribed into cDNA at 42 °C for 1 hour in 20 μl reaction mixture containing mouse Moloney leukemia virus reverse transcriptase (PrimeScript; Takara Bio) with random 6 primers (Takara Bio). Thereafter, generated cDNA was subjected to real-time PCR analysis using SYBR Premix Ex Taq II kit (Takara Bio) with the specific primers sets (Table 1 and Supplemental Table 1). Relative quantity of target gene expression to β-actin gene was measured by comparative Ct method.

ELISA for IFN-γ. Thrombus samples were obtained at the indicated time intervals and were homogenized with 0.3 ml of PBS (pH 7.2) containing Complete Protease Inhibitor Mixture (Roche Diagnostics). The homogenates were centrifuged at 12,000 g for 15 minutes. IFN-γ levels in the supernatant were measured using a specific ELISA kit (R&D Systems), according to the manufacturer’s instructions. The detection limit was greater than 2 pg/ml. Total protein in the supernatant was measured with a commercial kit (BCA Protein Assay Kit; Pierce) using BSA as a standard. The data were expressed as IFN-γ (ng/ml)/total protein (mg/ml) for each sample.

Gelatin SDS-PAGE substrate zymography. The intrathrombotic activities of MMP-2 and MMP-9 were determined by the Gelatin-Zymography Kit (Primary Cell) according to the manufacturer’s instructions. The data were expressed as band intensity/μg total protein.

Cell culture. WT mice were i.p. injected with 2 ml of 3% thioglycolate (Sigma-Aldrich), and i.p. macrophages were harvested (macrophage purity > 95%) 3 days later as described previously (53). The cells were suspended in antibiotic-free RPMI 1640 medium containing 10% FBS and incubated in a 5% CO2 incubator at 37 °C in three 24-well cell culture plates. Two hours later, nonadherent cells were removed, and the medium was replaced. After the cells were stimulated with PMA (50 ng/ml), PMA (50 ng/ml) plus IFN-γ (500 U/ml), or PMA (50 ng/ml) plus IFN-γ (500 U/ml) plus AG490 (100 nM) for 24 hours, the cells were subjected to subsequent analyses.

Western blotting. The isolated macrophages were homogenized with a lysis buffer (20 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Triton X-100, 1 mM EDTA) containing Complete Protease Inhibitor Cocktail (Roche) and phosphatase inhibitor cocktails for serine/threonine protein phosphatases and tyrosine protein phosphatases (P2850 and P5726; Sigma-Aldrich) and were centrifuged to obtain lysates. The lysates (equivalent to 30 μg protein) were electrophoresed in a 10% SDS-polyacrylamide gel and transferred onto a nylon membrane. After the membrane was sequentially incubated with optimally diluted primary Abs and HRP-conjugated secondary Abs, the immune complexes were visualized using the ECL system (Amersham Biosciences). The band intensities were measured using NIH Image Analysis Software version 1.61 (NIH) and were calculated as the ratios to β-actin.

Statistics. The means and SEMs were calculated for all parameters determined in this study. Statistical significance was evaluated by using ANOVA or Mann-Whitney U test. P < 0.05 was accepted as statistically significant.

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