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Low-dose Warfarin Functions as an Immunomodulator to Prevent Cyclophosphamide-induced NOD Diabetes

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Warfarin has been used as an anticoagulant for a long time. Recently, the pleiotropic effect of warfarin has been investigated. As low-dose warfarin has been reported to have anti-inflammatory effect through suppression of IL-6 secretion and inhibit the immune-associated signal between Tyro3 and its ligand, Gas6, the effect of low-dose warfarin on autoimmune diabetes in NOD mice was examined. To investigate the anti-inflammatory effect of warfarin, IL-6 secretion by splenocytes was examined in the presence of various concentrations of warfarin. Low concentration of warfarin inhibited IL-6 secretion. mRNA expression of Rse, one of the Tyro3 receptor family members, and Gas6 were analyzed in NOD mice. It was detected in islets, splenocytes and bone-marrow derived dendritic cells. 0.25 mg/l or 0.50 mg/l of warfarin was orally administered to NOD mice as a cyclophosphamide-induced diabetes model. Oral administration of warfarin at much lower doses than those clinically used as an anticoagulant significantly reduced the degree of insulitis and diabetes incidence in this model. We previously demonstrated that anti-FasL Ab-treatment led to complete prevention of autoimmune diabetes in NOD mice. As Fas/FasL signaling is reported to be essential for cyclophosphamide-induced diabetes model, we extracted RNA from lymphocytes of the inguinal lymph nodes of anti-FasL Ab-treated NOD mice and performed real-time PCR to determine expression of Rse gene. Interestingly, the expression of Rse gene related to the blockade of Fas/FasL signaling was reduced to less than half the level of untreated mice. In conclusion, low-dose warfarin is a potential immunomodulator which can prevent autoimmune diabetes.

Type 1 diabetes is a chronic autoimmune disease caused by autoreactive T cells promoting the specific destruction of insulin-producing β cells of the pancreatic islets (1,6). Nonobese diabetic (NOD) mouse is an animal model of human autoimmune diabetes (19). In the NOD mouse, diabetes develops as the result of a chronic inflammation that starts with leukocytic infiltration of islets from 3-5 weeks of age and gradually exacerbates until hyperglycemia develops after 16 weeks of age in a high percentage of female mice.

Warfarin has been widely used for a long time as an oral anticoagulant agent. In addition, Kater et al. reported the pleiotropic effect of low-dose warfarin related with inflammation, demonstrating that low-dose warfarin inhibited inflammatory signal transduction through suppression of TNF-α induced IL-6 secretion from murine macrophages (12).

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Warfarin also has been reported to inhibit the Tyro3/Gas6 signaling pathway (35). Yanagita et al. reported that Gas6 and Axl, one of the Tyro3 receptor members, play a key role in mesangial cell proliferation in the development of glomerulonephritis (38-40). This group also found that glomerular hypertrophy in diabetic nephropathy was inhibited by blockade of the Gas6/Axl-mediated pathway by means of oral administration of low-dose warfarin (23).

The Tyro3 receptor family, which include Axl, Rse (also known as Tyro3) and Mer were identified as a receptor tyrosine kinase subgroup in 1991 (14,28), and their two ligands, Gas6 and protein S, were identified and cloned four years later (34,36). Gas6 was first isolated as a gene product inducibly expressed in fibroblasts in response to growth arrest due to serum starvation. Gas6 has a structure similar to that of protein S, a vitamin K-dependent plasma protein, with an anticoagulant activity of 43% amino acid identity (20). In addition, Gas6 binds and activates all three receptors, but with markedly different affinities, namely, Axl>Rse>>Mer (24). However, Gas6 is functional only in fully γ-carboxylated form of glutamate residues at its N terminus in the presence of vitamin K. Warfarin inhibits γ-carboxylation of Gas6 through deprivation of vitamin K, so that the Tyro3/Gas6 signaling pathway is inhibited by warfarin (25,35).

The Tyro3 receptor family members are, together with their ligands, Gas6 and protein S, widely expressed in mammalian cells of the immune, nervous, vascular and reproductive systems (14,17,18). With reference to the immune system, Tyro3 receptors are prominently expressed by antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages (8,27), and play an essential immunoregulatory role in, for example, the regulation of the activation state of macrophages (15), or the rapid clearance of apoptotic cells by macrophages (31). Triple mutant mice that lack Tyro3 receptors develop a severe lymphoproliferative disorder accompanied by broad-spectrum autoimmunity (18).

In view of these findings, we focused on immunomodification of the diabetes process in NOD mice by means of low-dose warfarin, which may play a regulatory role in the immune system. Interestingly, oral administration of low-dose warfarin resulted in prevention of cyclophosphamide-induced diabetes in NOD mice.

MATERIALS AND METHODS

Mice. NOD/Shi/Kbe mice were kept at the Institute for Experimental Animals, Kobe University School of Medicine, Kobe, Japan. Insulitis becomes noticeable in most mice of our NOD colony at 5-7 weeks of age and is greatly enhanced with advancing age. The cumulative incidence of diabetes is 82.5% in females and 20.3% in males at 40 weeks of age. All animals were handled under the Guidelines for Animal Experimentation of Kobe University School of Medicine.

Isolation of Splenocytes and Pancreatic Islets. Splenocytes were isolated by dissection and filtered through a mesh strainer, followed by removal of erythrocytes with red cell lysis buffer. Pancreatic islet isolation was performed as described previously (21). Briefly, the common bile duct was identified, cannulated, and then injected with a solution of collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (1 μg/ml in HBSS). The digestion was performed for 20 min at 37ºC. The islets from six NOD male mice were washed in HBSS and filtered through a mesh strainer. The washed pellet was diluted in 10 ml Histopaque 1077 (Sigma, St. Louis, MO), overlaid with HBSS, and centrifuged. The resuspended islets were then hand-picked under a dissecting microscope.

Isolation of Bone Marrow (BM)-derived Dendritic Cells (DCs). Bone marrow-derived dendritic cells (BMDCs) were generated from BM cells derived from a 6-7-week-old NOD

mouse. In brief, BM was flushed from the femurs and tibias of the mouse, the BM cells were depleted of red cells and the remaining cells were seeded into 24-well plates at 1×10^6 cells/ml. They were cultured in the presence of 10 ng/ml GM-CSF (PeproTech EC Ltd., London, UK) for 6 days. On day 6, non-adherent and loosely adherent cells and clusters were harvested and CD11c+ cells were sorted with a magnetic cell sorter autoMACS (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) as BMDCs. The purity of CD11c+ cells was more than 95%.

IL-6 Production by LPS. Splenocytes were obtained from 8-week-old female NOD mice (n=6). Cells were cultured at 1×10^6 cells/well in 0.5 ml RPMI 1640 medium (10% FBS) in 24-well plates. In the presence of 100 ng/ml of lipopolysaccharide (LPS) or media alone, supernatants were collected 20h after stimulation with LPS for cytokine analysis. IL-6 ELISA was performed according to the manufacturer's protocol (GE Healthcare UK Ltd., Buckinghamshire, UK.).

Analysis of Rse and Gas6 mRNA Expression. Total RNA was extracted from islets, splenocytes and BMDCs with the GTC-phenol method using TRISOL. Pancreatic islets were isolated from six NOD mice and mixed together with an isolation procedure. After 5-10 $\times$ 10^6 splenocytes and BMDCs had been isolated from each mouse, cDNA synthesis was performed by using a cDNA cycle kit (Invitrogen, Carlsbad, CA) for reverse transcription, followed by PCR using Premix Taq (Takara Bio Inc., Shiga, Japan). The following primers were used for the PCR: 5'-GTGTGTGTGGCTGATTTTGG-3' and 5'-CATGATCTCCCAC ATGGTCA-3' for mRNA of Rse, resulting in a product of 174 nucleotides, and 5'-TGAGCTGCAGCTTCGGTACAA-3' and 5'-CATAGCCACTAAAGACGTGGG-3' for mRNA of Gas6, resulting in a product of 835 nucleotides (38).

Acceleration of Diabetes by Administration of Cyclophosphamide. Cyclophosphamide (Shionogi & Co., Ltd., Osaka, Japan) was dissolved in 0.9% NaCl at 20 mg/ml immediately before injection. Warfarin-treated mice and control mice were injected intraperitoneally with 300 mg/kg cyclophosphamide on days 0 and 14. All mice were nondiabetic before treatment.

Administration of Warfarin. Pre-diabetic 8-9-week-old male NOD mice in cyclophosphamide-induced diabetes model, were given 0.25 mg/l and 0.50 mg/l of warfarin potassium (Eisai Co., Ltd., Tokyo, Japan) in drinking water from day -7 to day 42 (10,41). Female NOD mice representing a spontaneous autoimmune diabetes model were given 0.25 mg/l and 1.00 mg/l of warfarin during 3-30 weeks of age. Warfarin was administered by adding the required volume of 25 mg/ml stock solution to distilled water. The drinking water was replaced with freshly diluted warfarin every 3-4 days. Water intake was monitored twice a week throughout the study. Mice were screened for glycosuria with the U-test Visual (Toyo Roshi Kaisha Ltd., Tokyo, Japan) twice a week, and if detected, diabetes was confirmed by blood glucose measurement using the Glutestsensor (Sanwa Kagaku Kenkyusho Co., Ltd., Nagoya, Japan). Mice with blood glucose of more than 250 mg/dl on 2 consecutive days were considered diabetic.

Histology. For the histological examination, mice were sacrificed 6 weeks after cyclophosphamide injection, and their pancreata were excised, fixed in 10% formalin, embedded in paraffin, and sectioned at a thickness of 5 μm. The pancreatic sections were then stained with hematoxylin/eosin, and 30-40 islets per individual pancreas were analyzed and scored for insulitis. Islets were categorized as 0 (intact islets), 1 (mild peripheral insulitis not exceeding 10% of the total islet), 2 (10-25% insulitis), 3 (25-50% insulitis), and 4 (more than 50% insulitis). The insulitis score was calculated with the formula: sum of (number of islets in each category $\times$ category) / total number of islets observed.

cDNA Array Analysis. Anti-FasL Ab-treated and untreated NOD mice were immunized with injection into their footpads of 100 μg insulin B9-23 peptide together with incomplete Freund's adjuvant (IFA) at 18 and 20 weeks of age. Inguinal lymph nodes from the anti-FasL Ab-treated-treated and untreated mice were crushed and dissolved in TRIZOL (Gibco BRL, Gaithersburg, MD). Total RNA was then extracted from the inguinal lymph nodes cells, and aliquots of RNA were used for gene expression analysis of immune-related molecules by Toyobo Gene Analysis Co., Ltd. (Osaka, Japan). Radioactive signals were detected with an FAS-1000 (Toyobo Co., Ltd., Osaka, Japan) and quantified. The signal intensity was normalized to the mean signal intensity of all features on an individual array. A more than 3-fold increase in signal intensity was considered significant.

Real-time PCR. The quantitative real-time PCR was performed on a LightCycler (Rosche Molecular Biochemicals, Mannheim, Germany) using the following primers for Rse: 5'-GTGTGTGTGGCTGATTTTGG-3' and 5'-CATGATCTCCCACATGGTCA-3' with SYBR Green. PCR was subjected to 120s of 95ºC hot-start enzyme activation, and 45 cycles of 98ºC denaturation for 2s, 55ºC annealing for 5s and 7ºC extension for 10s.

Statistical Analysis. The Kaplan-Mayer method was used for statistical analysis of diabetes incidence. $P < 0.05$ was considered significant. Nonparametric Mann-Whitney U test was used between two groups.

RESULTS

Effect of Warfarin on Cytokine Secretion. Since a previous study has shown that low-dose warfarin reduces IL-6 production, IL-6 secretion by splenocytes of NOD mice was examined in the presence of various concentrations of warfarin. IL-6 secretion by splenocytes was significantly reduced with 0.1 μM warfarin, which is much lower than the human serum concentration for clinical usage as an anticoagulant (4-5 μM), whereas IL-6 secretion was not inhibited with 5 μM warfarin, which is almost the same concentration as that used in clinical settings to suppress blood coagulation. On the other hand, IL-6 secretion was reduced much more with high concentrations of warfarin (100 and 200 μM) (Fig. 1).

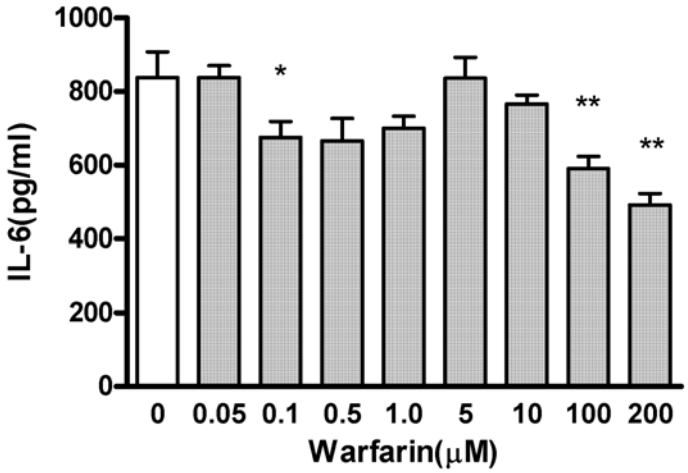

FIG.1. Warfarin inhibited IL-6 secretion *in vitro*.
Splenocytes obtained from 8-week-old female NOD mice were cultured with various concentrations of warfarin. After stimulation with 100 ng/ml of LPS, culture supernatants were collected and the IL-6 production was determined by means of ELISA. Data are presented as means ± SD (n=6) (*$p<0.05$, **$p<0.01$).

Expression of Rse and Gas6 in NOD Mice. We next examined whether Gas6 and one of its receptors, Rse, were expressed in NOD mice. Agarose gel electrophoresis showed that the reverse transcription-PCR products from islets, splenocytes and BMDCs in NOD mice had migrated to the predicted size of Rse and Gas6 cDNA fragments. Rse and Gas6 from islets and splenocytes were detected in NOD mice older than 13 weeks as well as in BMDCs generated from a 6-7-week-old NOD mouse (Fig. 2). These results suggested that Rse/Gas6 interaction was actively involved in the inflammation of NOD islets.

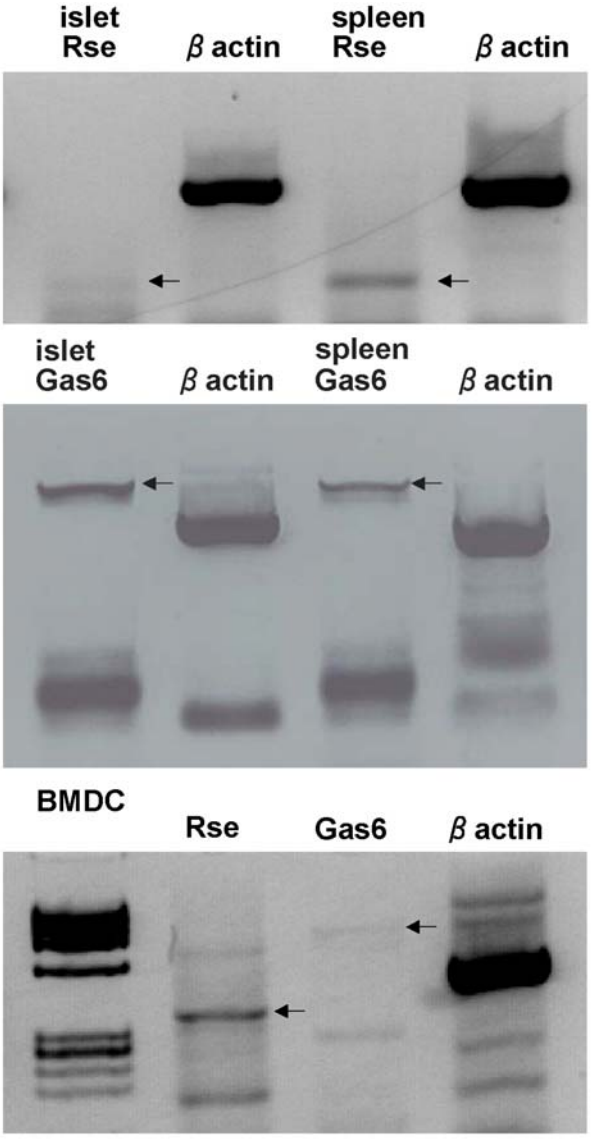

FIG.2. Rse and Gas6 were expressed in NOD mice.
NOD mice were subjected to reverse transcription-PCR. Rse and Gas6 from islets and splenocytes were detected in NOD mice older than 13 weeks. Both products were also detected in BMDCs generated from a 6-7-week-old NOD mouse.

Oral Administration of Warfarin to Cyclophosphamide-injected NOD Mice. To examine the effect of immunomodification of warfarin, 8–9 week-old male NOD mice were administered 0.25 mg/l and 0.50 mg/l warfarin in drinking water. Warfarin was administered from day -7 to day 42. Warfarin-treated and control mice were administered 300 mg/kg cyclophosphamide on days 0 and 14.

Administration of cyclophosphamide accelerated diabetes in all of warfarin-untreated NOD mice (n=15). Treatment with 0.25 mg/l warfarin (n=15) significantly reduced the incidence of cyclophosphamide-induced diabetes, while 0.50 mg/l warfarin (n=15) was not effective (27 and 73 %, respectively) (Fig. 3).

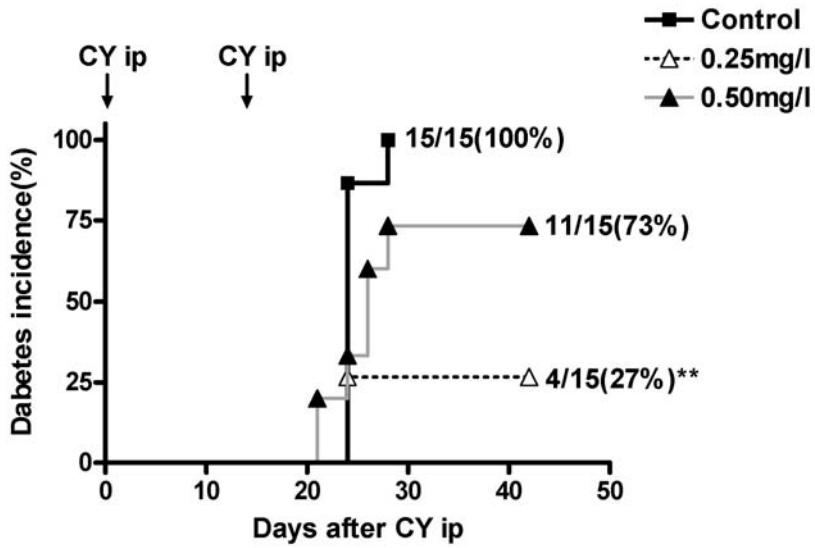

FIG.3. Development of cyclophosphamide (CY)-induced diabetes was significantly reduced by oral administration of low-dose warfarin.
8-9-week-old male NOD mice began to be administered two different doses of warfarin and were intraperitoneally (i.p.) given cyclophosphamide. 0.25 mg/l warfarin (open triangles, n=15) significantly reduced the incidence of cyclophosphamide-induced diabetes compared with that in untreated mice (control; squares, n=15) (27 vs. 100%, **p<0.01, Kaplan-Mayer method).

Reduced Insulitis after Warfarin Treatment. Assessment of the insulitis score for pancreatic sections showed conclusively that insulitis was prevented in 0.25 mg/l warfarin-treated group (n=8) compared with untreated mice (n=5) and 0.50 mg/l warfarin-treated group (n=7). These findings confirmed that autoimmune attack on β cells was minimal in low-dose warfarin-treated mice (Fig. 4).

Effect of Warfarin Treatment on Spontaneous NOD Diabetes. We also examined the effect of warfarin on spontaneous diabetes in NOD mice. To identify dose-dependent behavior of warfarin, 0.25 mg/l and 1.00 mg/l of warfarin were administered to female NOD mice from 3 weeks of age, and the development of diabetes was observed up to the age of 30 weeks. In untreated mice, diabetes occurred between 11 and 30 (median: 26.8) weeks of age and disease incidence at the end of the observation period was 80% (n=40). Mice administered 0.25 mg/l warfarin displayed a slightly delayed disease onset (median: 28 weeks, range: 14-30 weeks) with a diabetes incidence of 60%, though the difference was not statistically significant (n=20). Administration of 1.00 mg/l warfarin displayed diabetes incidence of 70% (median: 22.5 weeks, range: 17-30 weeks) (n=20)(Fig. 5).

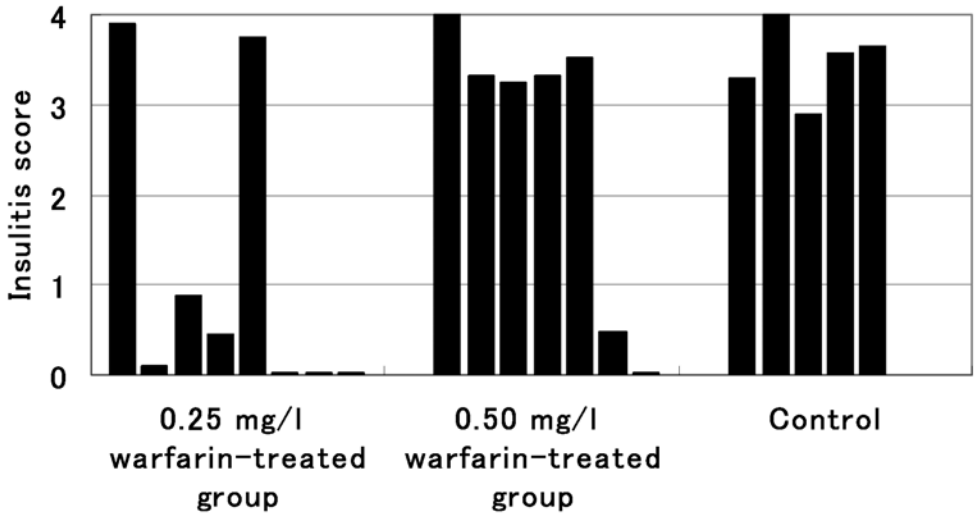

FIG.4. Warfarin administration reduced insulitis in cyclophosphamide-induced diabetes model.
Mice were sacrificed 6 weeks after cyclophosphamide injection. 30-40 islets per individual pancreas were analyzed and scored for insulitis. Insulitis was markedly prevented in 0.25 mg/l warfarin-treated group (n=8) compared with untreated mice (control, n=5) and 0.50 mg/l warfarin-treated group (n=7).

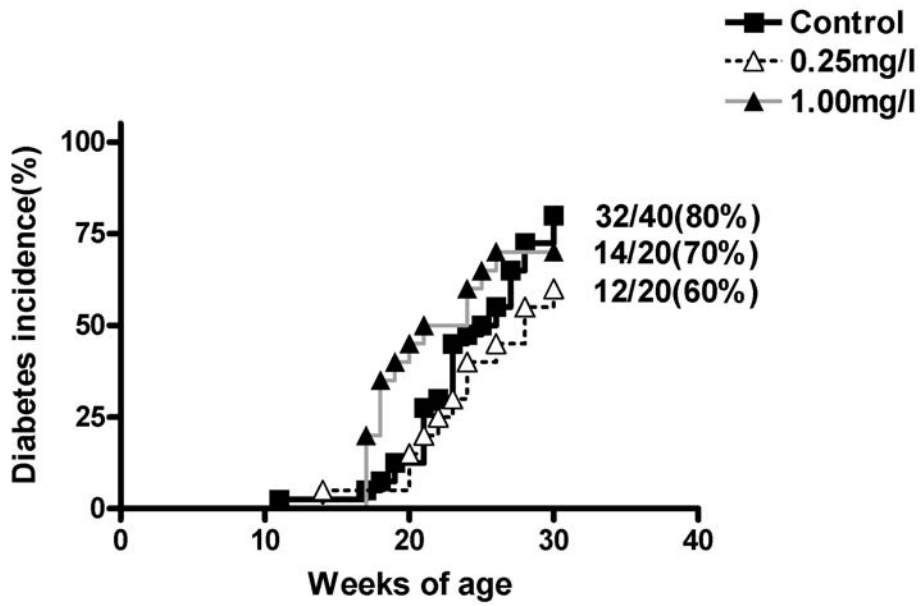

FIG.5. Development of spontaneous autoimmune diabetes was delayed and reduced by warfarin.
0.25 mg/l (open triangles, n=20) and 1.00 mg/l (closed triangles, n=20) of warfarin were administered orally from 3 weeks of age. NOD mice were observed up to 30 weeks of age. Mice treated with 0.25 mg/l warfarin displayed a delayed pattern and a reduced incidence of spontaneous autoimmune diabetes compared with untreated mice (control; squares, n=40), but not to a significant extent.

Expression of Rse Gene related to blockade of Fas/FasL signaling. In our previous study, blockade of Fas/FasL interaction by anti-FasL Ab led to complete prevention of insulitis and diabetes in NOD mice (26). Previous report has shown that Fas/FasL signaling is essential for the cyclophosphamide-induced diabetes model (2). To further investigate changes in gene expression related to blockade of Fas/FasL signaling, we extracted RNA from anti-FasL Ab-treated NOD mice and determined the relative expression level of 560 immune-related genes compared with those from untreated mice, by using cDNA array analysis. cDNA array analysis demonstrated that the gene expression level of Rse, one of the Tyro3 receptors, was significantly lower in the anti-FasL Ab-treated mice (Fig. 6).

To confirm this finding, we assessed the level of Rse gene expression in each inguinal lymph node by means of real-time PCR and found that Rse gene expression in the anti-FasL Ab-treated mice had decreased to less than half the level of that in untreated mice (Table 1).

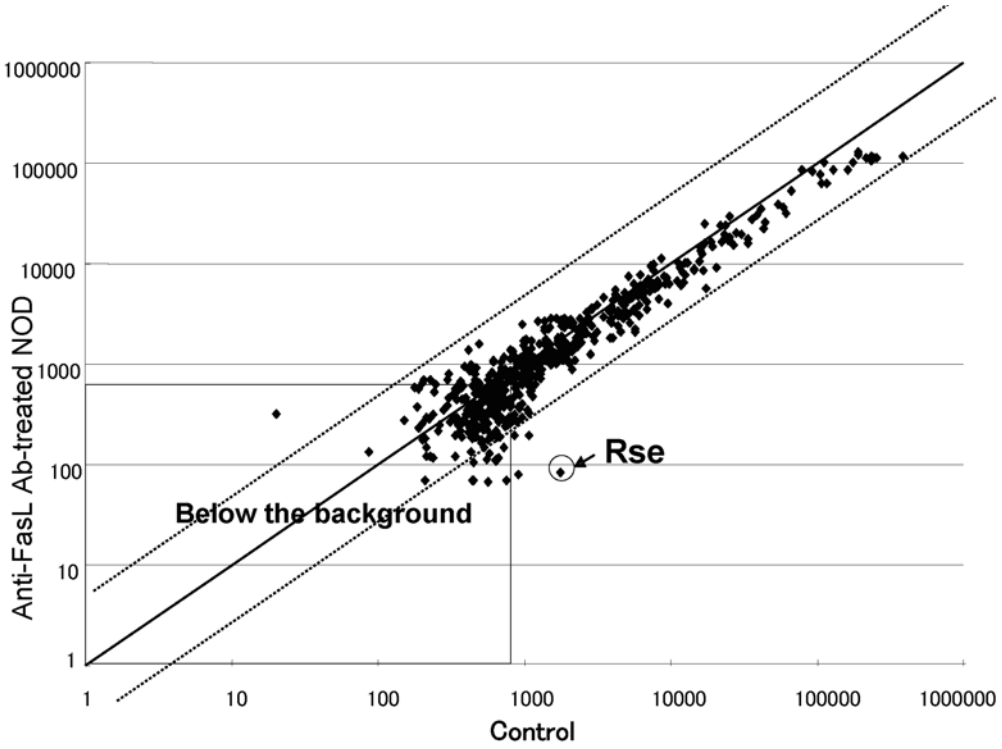

FIG. 6. The expression level of Rse gene was reduced in anti-FasL Ab-treated NOD mice.
cDNA array analysis was performed in inguinal lymph nodes from the anti-FasL Ab-treated NOD mice and untreated mice (control), which were immunized in advance with insulin B9-23 peptide and IFA in their footpads at 18 and 20 weeks of age.

TABLE 1. Rse gene expression in inguinal lymph nodes from anti-FasL Ab-treated NOD mice was detected at less than half level of untreated NOD mice by real-time PCR.

Sample	No. of calculated copies	No. of copies of Rse	SD
anti-FasL Ab	1.87×10^4	1.96×10^4	1.34×10^4
	2.06×10^4		
control	5.04×10^4	4.09×10^4	1.34×10^4
	3.14×10^4		

DISCUSSION

The novel finding of our study is that low-dose warfarin, functioning as an immunomodulator, can prevent autoimmune diabetes in NOD mice.

Warfarin has been widely used as an anticoagulant agent for a long time. In addition, its pleiotropic effect associated with inflammation has been reported so far. Kater et al. reported the observation of a dichotomal effect of warfarin on inflammatory signal transduction. Low-dose warfarin (<100 μM) inhibited TNF-α–induced IL-6 release from murine macrophages, whereas high-dose warfarin (100-250 μM) stimulated IL-6 release (12). IL-6 has been known to have a variety of functions including promotion of T cell activation (16), cytotoxic T cell differentiation (29) and maturation of activated B cells (22). Previous reports demonstrated that IL-6 produced in NOD islets might act as a costimulator for autoreactive B and T lymphocytes in autoimmune diabetes (4) and that administration of anti-IL-6 monoclonal antibody reduced the incidence of cyclophosphamide-induced diabetes in NOD mice (5). Indeed, the effect of low-dose warfarin to reduce IL-6 secretion was observed in our *in vitro* assay. As shown in Fig. 1, IL-6 secretion by splenocytes was significantly reduced by 0.1 μM warfarin, which was much lower than the clinical therapeutic concentration so that prothrombin time was not prolonged. However, IL-6 secretion was not inhibited by 5 μM of warfarin, which is almost the same as the therapeutic concentration for humans.

Warfarin is also known as an inhibitor of Tyro3/Gas6 signaling. Several studies have demonstrated that the Tyro3 receptor family members and their ligands greatly contribute to the immune system. Rse, Axl and Mer are prominently expressed on monocytes and their derivatives (8,27), and perform an essential function in the homeostatic regulation of APC activation (18). It has been shown that autoimmune diabetes might result from genetic defects manifest by APCs (32), and dendritic cells exhibit defects in function and maturation in NOD mice when compared to diabetes resistant strains (3,7,33,37). In the reverse transcription-PCR shown in Fig. 2, Rse and its ligand, Gas6 from islets and splenocytes were detected in NOD mice older than 13 weeks, suggesting that these products are derived from effector cells that have expanded in prediabetic mice. The detection of Rse and Gas6 in BMDCs indicates that these molecules are expressed on APCs in NOD mice. Nagai and his colleagues also found that glomerular hypertrophy in diabetic nephropathy was inhibited through blockade of the Gas6/Axl-mediated pathway by means of oral administration of low-dose warfarin (23).

In view of these findings, warfarin certainly effects the immune system involving IL-6 production or Tyro3/Gas6 pathway so that we wanted to modify the autoimmune diabetes process in NOD mice by means of low-dose warfarin. Administration of low-dose warfarin to cyclophosphamide-injected NOD mice actually resulted in a decrease of diabetes incidence as shown in Fig. 3. The dose of warfarin was determined on the basis of previous reports. Briefly, since mice drink on average 4.5 ml water/day, the administered doses of

warfarin, 0.25 mg/l and 0.50 mg/l, are equivalent to 0.036 mg/kg body weight/day and 0.072 mg/kg body weight/day, respectively, for mice weighing 20 grams. These doses are significantly lower than the usual therapeutic concentration of warfarin administered as an anticoagulant (4-5 μM) so that prothrombin time is not affected (9,11,13,30). Neither bleeding tendency nor anemia was observed in the warfarin-treated mice in our experiment.

Our *in vitro* findings are compatible with the narrow window of the effective dose of warfarin observed in our *in vivo* experiments. To our interest, our *in vivo* results presented in cyclophosphamide-induced diabetes model are consistent with the report that IL-6 inhibition reduced diabetes incidence in cyclophosphamide-induced model (5). Our results indicate that diabetes could be prevented if the proper concentration of warfarin is attained, and that this concentration may be much lower than the one used clinically.

In the NOD mouse, but not in other nondiabetes-prone strains, administration of cyclophosphamide accelerates diabetes at a young age when no spontaneous diabetes occurs (10,41). Balasa et al. reported that cyclophosphamide injection failed to provoke autoimmune insulitis and diabetes in Fas-deficient NOD-lpr/lpr (–/–) mice, while NOD-Fas/lpr (+/–) mice became diabetic, indicating that Fas expression is a prerequisite for cyclophosphamide-induced diabetes (2). We have previously shown that administration of anti-FasL Ab to female NOD mice during 2-4 weeks of age completely prevented insulitis and diabetes (26). To further examine this mechanism, we performed cDNA array analysis on the anti-FasL Ab-treated NOD mice in which Fas/FasL signaling was blocked and found that gene expression of Rse, one of Tyro3 receptors, was reduced as shown in Fig. 6. Real-time PCR confirmed that the expression of Rse in the anti-FasL Ab-treated mice was decreased to less than half the level of that in untreated mice (Table 1). Warfarin has been reported to block Tyro3/Gas6 signaling and we demonstrated that warfarin prevented cyclophosphamide-induced diabetes which is Fas/FasL-dependent model. We also found that the anti-FasL Ab-treated NOD mice in which Fas/FasL signaling is blocked presented reduced expression of Rse (Tyro3) gene. Taken together, prevention of cyclophosphamide-induced diabetes by means of low-dose warfarin might be in some way associated with the blockade of Rse (Tyro3)/Gas6 signaling and Fas/FasL signaling in addition to suppression of IL-6 production.

In spite of our findings concerning the efficacy of warfarin in the cyclophosphamide-induced diabetes model as shown in Fig. 5, warfarin could not significantly prevent spontaneous autoimmune diabetes, but delay diabetes onset. This may reflect diversity of pathogenesis among various subtypes of autoimmune diabetes. The precise mechanism, however, remains to be solved and further study still need to be done.

In summary, the administration of low-dose warfarin reduced the incidence of cyclophosphamide-induced diabetes. Low-dose warfarin warrants consideration as a potential immunomodulator which can prevent type 1A diabetes.

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