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Comparison of therapeutic efficacy between bortezomib and combination treatment of prednisolone and mycophenolate mofetil on nephritis in NZB/WF1 mice

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ABSTRACT

Objectives. *We investigated the efficacy of bortezomib on nephritis in NZB/WF1 mice, and compared it to prednisolone (PL) and mycophenolate mofetil (MMF) treatment.*

Methods. *Twenty-three NZB/WF1 mice were divided into four groups (untreated, 0.1 mg/kg and 1 mg/kg bortezomib-treated, and PL plus MMF-treated). Proteinuria, glomerular IgG deposition, cellular proliferation and histologic damages were evaluated.*

Results. *In comparison with the untreated mice, 1 mg/kg bortezomib significantly reduced proteinuria and attenuated glomerular IgG deposition, cellular proliferation and histologic damages similar to PL plus MMF. 0.1 mg/kg bortezomib significantly improved glomerular IgG deposition and histologic damages except proteinuria or cellular proliferation compared to untreated mice. PL plus MMF showed significantly greater inhibition of cellular proliferation and histologic damage than 1 mg/kg bortezomib.*

Conclusions. *We found that 1 mg/kg bortezomib significantly improved lupus nephritis in NZB/WF1 mice. However, its efficacy did not exceed PL plus MMF treatment.*

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease with multiorgan involvement, a wide variety of manifestations and an unpredictable course. Almost half of SLE patients present with asymptomatic hematuria and proteinuria (1). In lupus nephritis, complement (especially C3) and anti-DNA antibodies are involved in the pathophysiology of the disease, and the level of proteinuria reflects the extent of involvement of peripheral glomerular capillary loops, which tends to be increased along with mesangial proliferation as well as the extent of membraneous nephropathy (1). The use of intravenous or oral prednisolone (PL) and either intravenous cyclophosphamide or oral mycophenolate mofetil (MMF) currently are recommended as a standard treatment for induction therapy for patients with class III and IV lupus nephritis (2).

A proteasome functions to induce ubiquitin-mediated proteolysis of intracellular apoptosis regulatory proteins (3). Bortezomib induces apoptosis and alters expression of cytokines, cell adhesion proteins, and angiogenesis (4). Furthermore, bortezomib inhibits NF-κB activation by blocking the degradation of the NF-κB inhibitor (I-κB) and causes up-regulation of proapoptotic regulators as well as downregulation of anti-apoptotic proteins (4, 5). Recently, it was reported that NF-κB is a major regulator in mesangial proliferation in lupus-prone mice, and bortezomib is protective against lupus nephritis (6, 7). However, to our knowledge, there has been no report to compare the therapeutic efficacy of bortezomib to oral PL plus MMF treatment.

To address these issues, we investigated the efficacy of bortezomib on kidney disease in NZB/WF1 mice, as a model of human SLE, and compared it to oral PL and MMF treatment.

Materials and methods

Animals

Female NZB/WF1 mice at 16 weeks of age were purchased from SLC (Hamamatsu, Japan), and housed in individual cages in a specific pathogen-free barrier facility under standard sterile conditions at Yonsei University. All animals were treated in accordance with the guidelines and regulations for the use and care of animals of Yonsei University, Seoul, Korea.

Treatment protocol for lupus-nephritis prone mice

According to the preliminary experiment, about thirty percent of the mice in the untreated group died during the experimental period. Considering the rate of death in untreated mice, we assigned eight NZB/WF1 mice to group 1 and five mice to the other groups (group 1 = control, group 2 = 0.1 mg/kg of bortezomib-treated, group 3 = 1 mg/kg of bortezomib-treated and group 4 = oral PL plus MMF-treated). Treatment with bortezomib (Velcade, Millennium Pharmaceuticals, Cambridge, MA), or PL plus MMF treatment began at 24 weeks of age. One cycle of bortezomib treatment included the intraperitoneal

injections of bortezomib twice a week for the first 2 weeks and observation without intervention for one subsequent week. Four cycles of bortezomib treatment were completed sequentially over 12 weeks. Mice in group 4 received oral doses of 1 mg/kg PD and 17 mg/kg MMF daily during the bortezomib treatment period. Untreated and PL plus MMF-treated mice received intraperitoneal injection of PBS with the same frequency as bortezomib treatment.

Measurement of proteinuria

Proteinuria was measured in spot urine collected from each mouse using an albumin reagent strip (URiSCAN; Yongdong Pharmaceutical Co., Seoul Korea) twice weekly during the experimental period. Proteinuria was semi-quantitatively expressed as follows: 0 = none or trace, 1+= less than 100 mg/dL, 2+= less than 300 mg/dL, 3+= less than 2000mg/dL, and 4+= more than 2000mg/dL.

Histopathologic assessment

The mice were anesthetised and sacrificed at 36 weeks of age. Kidney specimens were obtained from all mice and fixed in formaldehyde, embedded in paraffin and sectioned at 4 micro-meter thickness. Kidney sections were stained with periodic-acid Schiff (PAS) and hematoxylin and eosin. Glomerular cells were counted using MetaMorph Version 5 (Molecular Devices Inc., Downingtown, PA) according to the manufacturer's protocol. Also scoring for renal histologic abnormalities of mice with nephritis including glomerular, tubular and vascular damages was semi-quantitatively performed on a four-point scale independently and blindly by two individual pathologists, and the averages of their scores were calculated. A score of 0 represented no changes, a score of 1 represented mild changes, and a score of 2 represented moderate changes, whereas a score of 3 represented marked changes (7, 8). At least 50 glomeruli were examined per mouse.

Immunohistochemical staining

To detect immunoglobulin G (IgG) deposition in glomeruli, all kidney sections were stained with specific antibodies directed against murine IgG (Sigma; St. Louis, MO) and appropriate secondary antibodies (ISU Abxis, Seoul, Korea). All tissue samples were counterstained with hematoxylin. Scoring for IgG deposition was semi-quantitatively performed on a four-point scale independently and blindly by two individual pathologists, and the averages of their scores were calculated. A score of 0 represented no deposits, a score of 1 represented mild deposition, and a score of 2 represented moderate deposition, whereas a score of 3 represented intense deposition. At least 30 glomeruli were examined per mouse.

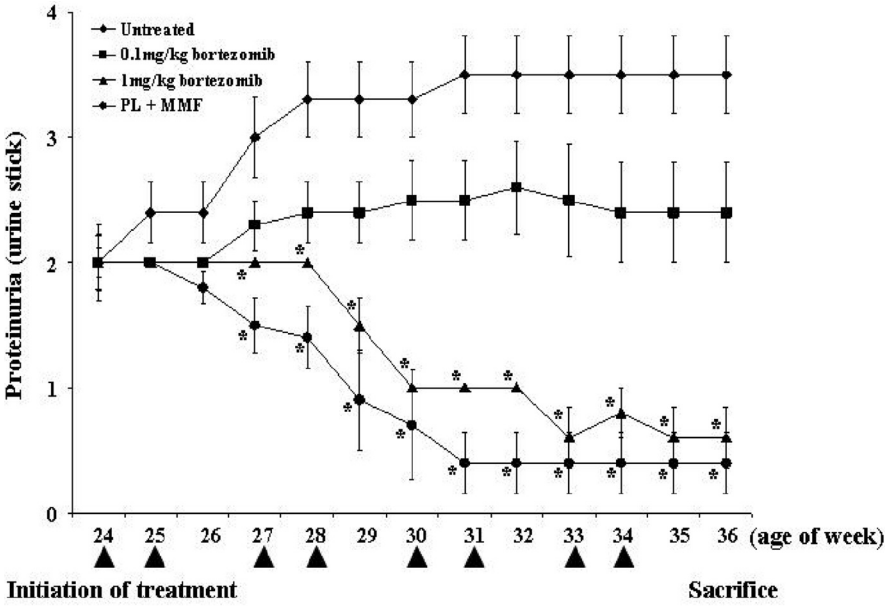

Fig. 1. Proteinuria in NZB/WF1 mice. In comparison with untreated mice, 1 mg/kg bortezomib as well as prednisolone (PL) plus mycophenolate mofetil (MMF) significantly reduced proteinuria by 83% and 89%, respectively. Treatment with 0.1 mg/kg bortezomib also decreased proteinuria by 31% compared to untreated mice, but this was not significant (*p-value<0.05). Injection of bortezomib was represented by (▲).

Statistical analysis

All statistical analyses were conducted using the SPSS package for Windows, Version 13 (SPSS Inc., Chicago, IL). The representative values were the means of those obtained from each mouse in each group, and all values in the experimental groups were compared to untreated mice. All results and measurements are expressed as the mean ± standard deviation. Statistical comparisons of proteinuria, IgG deposition and histologic damages between the two groups were evaluated by Mann-Whitney U test. Glomerular cell counts were compared using Student's t-test. When we compared the values between untreated and each treated groups, we used the asterisk (*) to designate the mean value of each treated group that had statistical significance (p-value <0.05).

Results

Proteinuria in NZB/W F1 mice

Three mice died in group 1 and five mice alive were compared to those in the other treated groups. Fig. 1 shows proteinuria in NZB/WF1 mice during the experimental period. At 24 weeks of age, most mice showed 2+ proteinuria. Proteinuria gradually increased and reached 3.5+ in untreated mice at the end of the experimental period. At 36 weeks of age, in comparison with proteinuria in untreated mice, 1 mg/kg bortezomib as well as PL plus MMF significantly reduced proteinuria by 83% and 89%, respectively. Comparatively, 0.1 mg/kg bortezomib decreased proteinuria by 31% compared to untreated mice, but the difference was not significant.

Glomerular deposition of IgG

Immunohistochemical studies showed intense glomerular staining for IgG

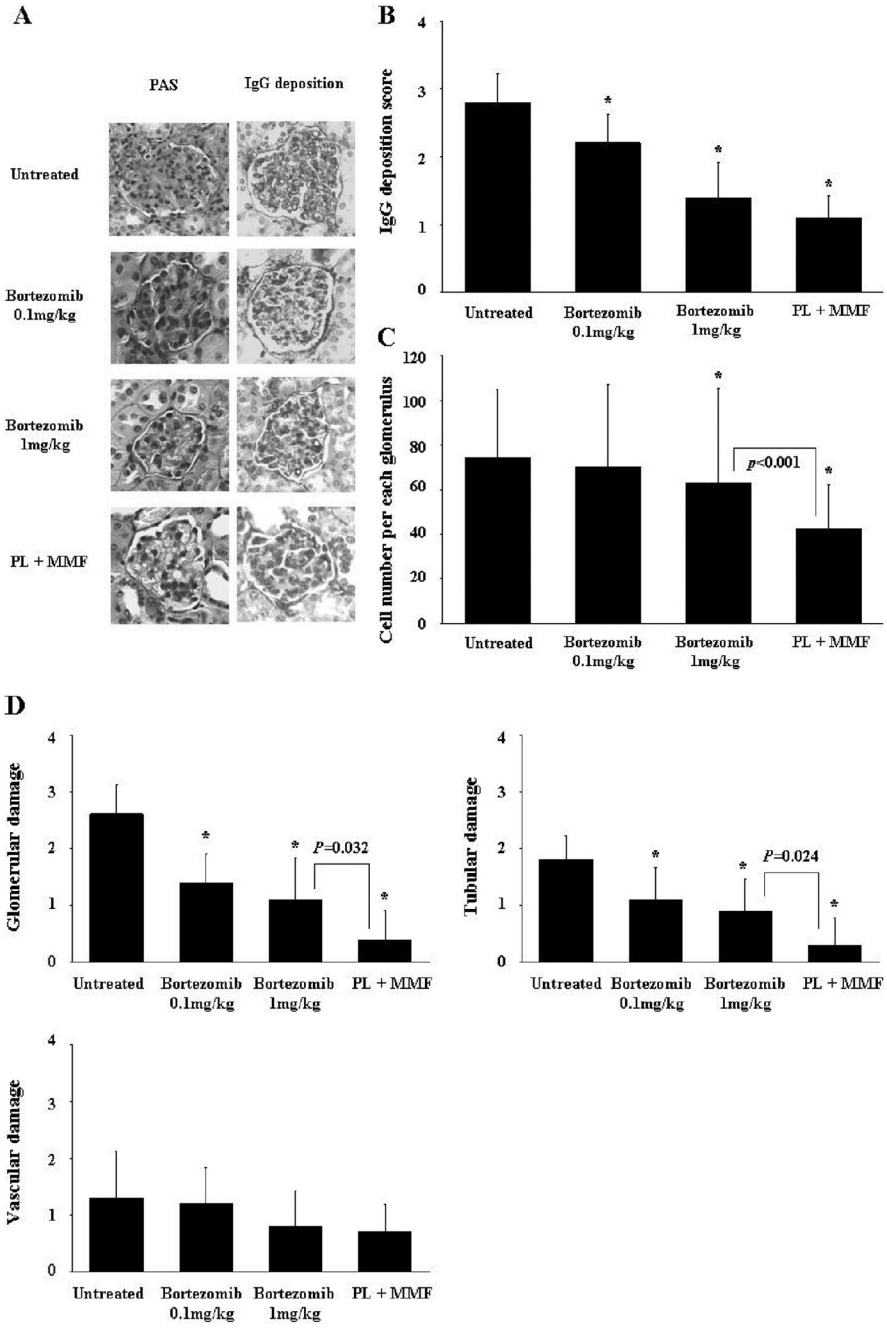

Fig. 2. IgG deposition and cellular proliferation in glomeruli. Hypercellularity and intense IgG deposition in glomeruli were observed in untreated mice, while relatively low cellular proliferation and weak IgG deposition in glomeruli were observed in mice treated with bortezomib as well as prednisolone (PL) plus mycophenolate mofetil (MMF) (**A**). Bortezomib and PL plus MMF resulted in a significant decrease in glomerular IgG deposition compared to untreated mice (**B**). In comparison with untreated mice, 1 mg/kg bortezomib as well as PL plus MMF reduced cell proliferation, while, 0.1 mg/kg bortezomib resulted in no significant difference. PL plus MMF resulted in significant inhibition of cellular proliferation in glomeruli than 1 mg/kg bortezomib (**C**). Histologic evaluation showed that 0.1 mg/kg bortezomib, 1 mg/kg bortezomib and PL plus MMF significantly decreased glomerular damage score as well as tubular damage score. Particularly, PL plus MMF significantly reduced glomerular and tubular damage scores greater than 1 mg/kg bortezomib (**D**). (*p-value<0.05).

in untreated mice, whereas only weak staining for IgG was observed in bortezomib-treated as well as PL plus MMF-treated mice (Fig. 2A). Bortezomib significantly decreased glomerular IgG deposition compared to untreated mice (21% for 0.1 mg/kg and 50% for 1 mg/kg, respectively). A reduction of 60% in glomerular IgG deposition was observed in PL plus MMF-treated mice, in comparison with untreated mice (Fig. 2B).

The extent of cellular proliferation in glomeruli

Mean glomerular cell number in untreated mice was 74.1±30.6. For the treated mice, mean glomerular cell numbers were as follows; 70.2±36.6 for 0.1 mg/kg bortezomib, 62.8±42.2 for 1 mg/kg bortezomib and 42.0±20.1 for PL plus MMF, respectively. In comparison with untreated mice, 1 mg/kg bortezomib reduced glomerular cell number by 15%, PL plus MMF reduced glomerular cell number by 43%, which were significantly, and 0.1 mg/kg bortezomib showed no significant difference. Moreover, PL plus MMF significantly inhibited cellular proliferation in glomeruli greater than 1 mg/kg bortezomib (Fig. 2C).

Histologic abnormalities

Histologic evaluation showed that 0.1 mg/kg bortezomib, 1 mg/kg bortezomib and PL plus MMF significantly decreased glomerular damage score by 38%, 58% and 85% respectively as well as tubular damage score by 39%, 50% and 83%, respectively. However, there were no significant differences in vascular damage scores among the experimental groups. Particularly, PL plus MMF significantly reduced glomerular and tubular damage scores greater than 1 mg/kg bortezomib (Fig. 2D).

Discussion

In the present study, we found that bortezomib ameliorated the progression of lupus nephritis in NZB/W F1 mice in a dose-dependent manner. Bortezomib at a dose of 1 mg/kg significantly reduced proteinuria and attenuated IgG deposition and cellular proliferation in glomeruli. However, there were discrepancies in results from the 0.1 mg/kg bortezomib-treated mice; 0.1 mg/kg bortezomib significantly decreased glomerular IgG deposition, but showed no significant effect on proteinuria and cellular proliferation compared to untreated mice. We postulated that there might be a time lag between immune reaction and clinical outcomes; thus, 0.1 mg/kg bortezomib might mitigate

IgG production, but might not alleviate the severity of proteinuria or cellular proliferation due to uncorrectable structural changes resulting from the disease.

According to a previous study, 0.75 mg/kg bortezomib was efficient for the treatment of lupus nephritis (7). Although, 0.1 mg/kg bortezomib was not a sufficient dose for improving the clinical course of lupus nephritis in this study, it definitely altered immune reactions and showed significant therapeutic efficacy in another autoimmune disease model, collagen induced arthritis mice (9). Thus, the critical dose of bortezomib necessary to ameliorate lupus nephritis might range between 0.1 and 0.75mg/kg.

Diffuse proliferative glomerulitis, which is induced by immune complex deposition, is the most common type of lupus nephritis, and exhibits features of glomerular expansion due to proliferation of glomerular cells as well as crescent formation (10). Since the outcome of lupus nephritis has been shown to correlate with histopathologic changes such as glomerular hypercellularity, it is reasonable to count glomerular cells to reflect the extent of kidney damage in NZB/W F1 mice (11, 12). Cellular proliferation in glomeruli, however, has been evaluated by semi-quantitative methods to date. In this study, we directly counted glomerular cells using an automatic cell-counting system. To validate the correlation of cellular proliferation in glomeruli with renal histologic changes, we also assessed glomerular, tubular and vascular damage scores and found that the results except vascular changes showed the similar patterns to cellular glomerular proliferation.

Although 1 mg/kg bortezomib improved proteinuria and histopathologic features similar to standard treatment, its therapeutic potency did not exceed that of PL plus MMF. Specifically, PL plus MMF showed greater inhibition of cellular proliferation in glomeruli than bortezomib. Also, the dose of bortezomib used in this study is much higher the usual dose for multiple myeloma patients, 1.5 mg/m^2 per each time, which might induce serious side effects such as polyneuropathy, thrombocytopenia and gastrointestinal complications (13). Considering the expense and adverse effects of bortezomib, when a clinical trial is planned, further studies are necessary to clarify therapeutic efficacy and dose of bortezomib for the treatment of lupus nephritis.

In conclusion, 1 mg/kg bortezomib significantly reduced proteinuria and attenuated IgG deposition, histologic damages and cellular proliferation in glomeruli in NZB/W F1. However its efficacy did not exceed that of PL plus MMF treatment.

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