Pathogenic effect of a cell-penetrating anti-dsDNA autoantibody through p38 signaling pathway and pro-inflammatory cytokine stimulation in mesangial cells

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ABSTRACT

A subset of anti-dsDNA autoantibodies (autoAbs), cell-penetrating Abs, may play a pathogenic role in lupus nephritis. However, the pathogenic role(s) of the Abs has not been well explored. In this study the pathological effects of a positively charged CDR3-VH-containing and cell-penetrating anti-dsDNA monoclonal mouse autoAb 2C10 immunoglobulin G (IgG) and its recombinant VH domain were investigated in a mouse mesangial cell line with respect to activation of signaling molecules and transcription of pro-inflammatory cytokines. The IgG and VH reduced cell viability in cytotoxicity assays and delayed cell cycle progress in flow cytometric analysis. Western blotting experiments showed that they activated p38, MAPKAPK-2, RSK-1, Bcl-2 and ATF-2 in the associated pathway; RSK-1 activation was regulated by p38; p38 also activated MAPKAPK-2 and ATF-2; MAPKAPK-2 regulated RSK-1 activation, and Bcl-2 was up-regulated by RSK-1. The IgG and VH remarkably stimulated the transcription of pro-inflammatory cytokines, TNF-α, IL-6 and IL-1β. And the transcription was regulated by p38 activation. These results indicate that the cell-penetrating autoAbs such as 2C10 may play a pathogenic role in mesangial cells mainly through activation of p38 signaling pathway in combination with the stimulation of pro-inflammatory cytokine production.

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Introduction

Systemic lupus erythematosus (SLE) and lupus nephritis (LN), the severe and late stage manifestation of SLE, are a complex pathological process. Anti-double stranded DNA (dsDNA) autoantibodies (autoAbs) can be characteristically found in patients with SLE and LN. There is accumulating evidence for the pathogenic role of anti-dsDNA autoAbs in induction and progress of the diseases (Jang et al. 1996; Puttermann 2004; Isenberg et al. 2007; Im et al. 2015). It has been reported by different research groups including ourselves that some autoAbs have cell-penetrating properties, and this subset of Abs could be involved in pathogenesis (Alarcon-Segovia et al. 1978; Lee et al. 2007; Jang et al. 2009; Im et al. 2015). However, the mechanism(s) by which cell-penetrating autoAbs contribute to the diseases are not well understood. Recently, it has been reported that cell-penetrating anti-dsDNA autoAbs have multiple arginines in the complementarity determining region 3 of variable heavy domain (CDR3-VH) and one of the Abs, 2C10 IgG and its recombinant VH, penetrate cells (Im et al. 2015, 2017) and increase the levels of pERK1/2 and Bcl-2 in renal mesangial (MES) cells, the main target in LN (Im et al. 2015). 2C10 IgG has been produced from the SLE mouse model MRL-1pr/lpr (Stollar et al. 1986; Kubota et al. 1996; Im et al. 2015). The recombinant 2C10 VH single domain has been produced by engineering the structure of 2C10 IgG (Im et al. 2015, 2017). The penetrating autoAbs can amplify disease severity by affecting the target cells in various ways (Kubota et al. 1996; Sun et al. 2000; Yu et al. 2001; Lee et al. 2007; Jang et al. 2009; Im et al. 2015). Cytokines released by MES cells could lead to stimulation of other pro-inflammatory cytokines and inflammation of renal tissues, resulting in kidney damage (Graninger et al. 2000; Feng et al. 2012).

In this study, we aimed to explore signaling molecules in additional to ERK and Bcl-2 activated in MES cells treated with 2C10 IgG and VH domain. We found the activation of p38, MAPKAPK-2, RSK-1 and ATF-2, and the changes in transcription levels of pro-inflammatory cytokines by the activated p38. Decreased cell survival and delay of cell cycle were also caused by 2C10 IgG and VH. Our results strongly support the evidence for a possible mechanism of cell-penetrating anti-dsDNA autoAbs in MES cells and their environment in the pathogenesis of LN.
Materials and methods

Cell lines

SV40MES13 (mouse MES cell line), was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in specific cell culture media and grown at 37°C in a CO2 incubator. Cell culture media was a 3:1 mixture of Dulbecco’s Modified Eagle’s Medium and Ham’s F12 medium (Gibco-BRL, Rockville, MD, USA) with 14 mM HEPES, 10% FBS and 5% penicillin.

Anti-dsDNA monoclonal antibodies

2C10 and G5-8 IgGs were obtained from the mouse model MRL-lpr/lpr (im et al. 2015). 2C10 VH domain was produced from 2C10 IgG by recombinant technology (im et al. 2017).

Cell signaling antibodies and inhibitors

Abs for ERK1/2, pERK1/2, p38, pMAPKAPK-2, pMAPKAPK-2, ATF-2, pATF-2, BCL-2 were purchased from Cell signaling technologies (Danvers, MS, USA). Abs for RSK1 and pRSK1 were from SantaCruz (Dallas, TX, USA). U0126 and PD98059 (ERK1/2 inhibitors) and SB203580 (p38 inhibitor) from Cell signaling technologies. PF3644022 (MAPKAPK-2 inhibitor) (TOCRIS, Bristol, United Kingdom), LJI308 (RSK inhibitor) (Sigma Aldrich, St. Louis, MI, USA) were used for analysis of the signal molecules. Anti-mouse IgG-HRP and Anti-rabbit IgG-HRP were used for secondary Abs. Dilution ratios of Abs and inhibitor concentrations as defined by the product data sheets were used for Western Blot assay.

Cytokine primers

Pro-inflammatory cytokine primers were purchased from COSMO Gentech (Seoul, South Korea).

TNF-α

Forward (FW): 5’-CTC CAG GCG GTG CCT ATG T-3’
Reverse (RV): 5’-GAA GAG CGT GGT GGC CC -3’.

IL-6

FW: 5’ – CCA GAA ACC GCT ATG AAG TTC C -3’
RV: 5’- TCA CCA GCA TCA GTC CCA AG -3’.

IL-1β

FW: 5’-TCC AGG ATG AGG ACA TGA GCA C-3’
RV: 5’- ACC TGC TCC ACT GCC TGG CT -3’.

Glucuronidase beta (GUSB)

FW: 5’- TCC GTA TGT GGA TGT CAT GTG T- 3’
RV: 5’- ATC AGA GGT GGA TCC TGG TG -3’.

Cell viability analysis

Cell survival rate was analyzed by using the EZ-CyTox assay kit (DoGen, Seoul, Korea).

Flow cytometry for cell cycle analysis

For the cell-cycle analysis, cells were harvested in 6-well plates. 50 μg/ml of 2C10 IgG, VH Domain and G5-8 IgG were applied to the cells, then incubated at 37°C for 24 h. Cells were collected by using 0.05% trypsin and 0.02% EDTA and centrifuged, supernatant removed by pipetting, then 70% ethanol (1 ml) was added and incubated O/N at 4°C. Next, cells were washed with PBS and 0.5 mg/ml of RNase was added and incubated 1 h at 37°C. Finally, 30 μg/ml PI solution was added and samples were examined in FACS Canto II apparatus (Becton-Dickinson, Franklin Lakes, NJ, USA) with FlowJo-7 software.

Western blotting

For the analysis of the signal molecules, cells were harvested in a 12 well plate. 100 μg/ml of Ab treatments were applied and incubated from 30 min to 24 h. Cells were lysed by using RIPA buffer and incubated 1 h at 4°C. Samples were collected and added to 5X sample buffer in each sample, then heated at 95°C for 5 min. Samples were loaded in 10% SDS-gel and proteins blotted on nitrocellulose paper. Specific and appropriate secondary Abs were used as directed by the product data sheet. ECL reagent (Amersham Life Science, Piscataway, NJ, USA) was applied on the membrane for visualizing bands on film.

Quantitative real-time PCR (qRT-PCR)

Cells were seeded in 6 well plates, treated with 100 μg/ml of Abs and incubated for 3, 6, 12 and 24 h at 37°C. Cells were treated with 100 μg/ml of Abs and incubated for 3, 6, 12 and 24 h at 37°C. Cells were treated with 100 μg/ml of Abs and incubated for 3, 6, 12 and 24 h at 37°C. Cells were treated with 100 μg/ml of Abs and incubated for 3, 6, 12 and 24 h at 37°C. Cells were treated with 100 μg/ml of Abs and incubated for 3, 6, 12 and 24 h at 37°C. Cells were treated with 100 μg/ml of Abs and incubated for 3, 6, 12 and 24 h at 37°C. Cell pellets were collected and total RNA extracted from the pellet (RNA- spin Kit, Intron Biotechnology, Sungnam, Korea). cDNA was synthesized by using TaKara Prime Script kit (Takara Bio, Otsu, Japan). 100 ng/ml of template and 0.2 μM of cytokine primers were added to 2X SYBR premix (Takara) containing 1X ROS. The primers for the house-keeping gene GUSB were used as internal normalization controls. Samples were analyzed in an Applied Biosystem 7000qPCR (Applied Biosystems, Waltham, MS, USA). For the analysis, the 2△△T method (Livak and Schmittgen 2001) was performed.
**Statistical analysis**

All results were shown as mean ± standard deviation. The t-test was used for statistical analyses of data from two to five independent experiments. The significance of differences from control groups was shown as values of probability and indicated as asterisks; *p < 0.05, **p < 0.005.

**Results**

**2C10 IgG and VH domain decreased cell survival and delayed the cell cycle progress**

Our group previously reported the cell-penetrating 2C10 IgG and VH domain, not the non-penetrating anti-dsDNA G5-8, reduced survival of MES cells in a MTT assay (Im et al. 2015). To confirm the inhibition of cell survival, we performed EZ-Cytox assays (Figure 1(A)) and analyzed cell cycle phases with the IgGs and 2C10 VH domain by flow cytometry (Figure 1(B)). Cell viability was decreased by 25 or 50 μg/ml of IgG and VH of 2C10. With 50 μg/ml of Abs, viability was about 78% after 24 h. After cells were treated with 100 μg/ml of the Abs for 24 h, the ratios of G0/G1+S+G2M were calculated from the percentages for each cell cycle phase. An increase in the ratio represents a reduction of cell proliferation or survival. An increase in the ratio of 45% was observed in cells treated with 2C10 IgG and 62% increase with VH, but no change was seen with G5-8 IgG. The ratio decreased 41% in cells treated with 2C10 IgG and VH domain, not the non-penetrating anti-dsDNA G5-8 IgG. The ratio decreased 41% in cells treated with excess thymidine (2 mM), which is the early S phase blocker (Jackman and O’Connor 2001). Treatment with 2C10 IgG and VH domain inhibited cell survival and impaired the progress of cell cycle.

**2C10 IgG and VH domain activated p38 and sequential signal molecules**

The effects of 2C10 IgG and VH domain on activation of cell signal molecules were analyzed by Western blotting. Cells were incubated with the Abs for different periods of time (30 min, 1 h, 3 h, 6 h, 16 h and 24 h) at 37°C. p38 was activated from 30 min to 24 h after treatment with 2C10 IgG and VH (Figure 2(A)). However, p38 was not significantly activated by G5-8 IgG until 24 h. Phosphorylation of RSK-1 was observed at 6 h following VH treatment and 6 h to 24 h after treatment with IgG of 2C10. Previously, it was shown that ERK1/2 was also activated in MES cells by 2C10 IgG and VH (Im et al. 2015). Inhibitor studies were performed to explore the sequential activation of the molecules. To test the possible activation of RSK-1 by ERK1/2, the experiment was done using ERK1/2 inhibitors. PD98059 (40 μM) and U0126 (10 μM) had no effect on the activation level of RSK-1 (Figure 2(B)), although they suppressed the phosphorylation of ERK1/2.

Activation of MAPKAPK-2 and ATF-2 signaling molecules was observed from 30 min until 24 h after treatment with 2C10 IgG and VH (Figure 3(A)). The p38 inhibitor, SB203580, was examined to identify the downstream molecules activated by p38. SB203580 (10 μM) significantly reduced the levels of pMAPKAPK-2, pRSK-1, and pATF-2 (Figure 3(B)). For further analyses of the upstream activator of RSK-1, the MAPKAPK-2 inhibitor (PF3644022 10 μM) was used with IgG and VH (Figure 3(C)). The MAPKAPK-2 inhibitor suppressed RSK-1 activation, indicating that activation of MAPKAPK-2 regulates RSK-1 activation. Our previous reports (Im et al. 2015) showed that in MES, 2C10 IgG and VH domain up-regulated Bcl-2 and ERK. However, the ERK1/2 inhibitors (PD98059 and U0126) did not inhibit Bcl-2 levels (Figure 3(B)). Therefore, we tried to identify the upstream regulator of Bcl-2. We found that inhibitors for RSKs (RSK1–4), LJI308 (20 μM) dramatically downregulated Bcl-2 protein levels which were upregulated by 2C10 IgG and VH domain (Figure 3(D)). We confirmed that RSK-1 is the upstream activator of Bcl-2 in MES cells treated with 2C10 IgG and VH. Overall, 2C10 activates the p38-MAPKAPK2-RSK1-Bcl2 signaling pathway and ATF-2 signal molecule.

**2C10 IgG and VH increased the mRNA Level of TNF-α, IL-6 and IL-1β**

A quantitative real-time PCR (qRT-PCR) experiment was performed to determine the pattern of pro-inflammatory cytokine gene transcription in cells treated with 2C10 IgG, 2C10 VH and G5-8 IgG. Cells were treated with the Abs for different periods of time (3, 6, 12 and 24 h) at 37°C. 2C10 IgG and VH domain increased mRNA levels of TNF-α, IL-6 and IL-1β in a similar pattern to one another. The mRNA levels of the cytokines began increasing at 3 h, reached maximum levels at 6 h, decreased at 12 h and increased again at 24 h (Figure 4(A)). With 2C10 IgG, the increased transcription levels of TNF-α, IL-6 and IL-1β were 6-fold, 4.6-fold, and 10.9-fold at 6 h, respectively. At 6 h, 2C10 VH increased the levels by 6.2-fold, 4.8-fold, and 8.2-fold, respectively. G5-8 IgG did not affect the transcription levels of the three cytokines significantly. The protein levels of TNF-α and IL-6 increased 2 to 4-fold at 6 h (data not shown) with 2C10 IgG. More extensive work with 2C10 IgG and VH for analyzing the protein levels of the three cytokines should be performed and is proceeding. To prove a linkage between the activation of signaling molecules and the production of pro-inflammatory cytokines, cells were pre-treated with a p38 inhibitor (SB203580) for
Figure 1. Effects of 2C10 IgG and VH domain on cell survival and cell cycle phases. (A) Cells were incubated with 25 μg/ml and 50 μg/ml of IgGs and VH for 24 h at 37°C. The normal mouse IgG (mlgG) was used as a negative control. Cell survival was determined by the EZCytox assay. (B) 100 μg/ml of samples were treated to cells, and cells were cultured for 24 h at 37°C. Cell cycle phases were analyzed by flow cytometry, and the data were represented as the ratios of G0G1/S+G2M. Control represents cells without any treatment. Excess thymidine (2 mM) was used as the early S- phase blocker. The box represents the data for flow cytometric analysis from which the ratio of cell cycle phases was calculated. Each group’s p value was calculated. (*p < 0.05, **p < 0.005). The experiments of (A) and (B) were performed five times and twice, respectively.

Figure 2. Activation of p38, ERK1/2, and RSK-1 by 2C10 IgG and VH and effect of ERK inhibitor on RSK-1 activation. (A) Cells were incubated with 30 min to 24 h. Cell lysates were loaded in 10% SDS-PAGE and signal molecules analyzed by using appropriate Abs to specific signaling molecules. (B) Cells were pre-treated with inhibitors for 2 h, then incubated with Abs (100 μg/ml) for 6 h, and whole cell lysate samples were analyzed by Western blotting.
2 h, then incubated with Abs for 6 h for qRT-PCR analysis. Results showed that the p38 inhibitor totally blocked the enhanced transcription levels of the cytokines (Figure 4 (B)). These data indicate that 2C10 can stimulate the activation of pro-inflammatory cytokine genes under the regulation of p38.

Discussion

In the present study, we showed that the intracellular changes in MES cells were induced by a cell-penetrating 2C10 IgG and VH domain. In SLE, it has been known that special structures and properties in CDR3-VH are one of the factors conferring pathogenic properties to autoAbs (Raz et al. 1989; Jang et al. 1998; Jang and Stollar 2003; Lambrianides et al. 2008; Im et al. 2015). 2C10 contains three positively charged arginines in the CDR3-VH region (Jang et al. 1996; Im et al. 2015). Other known autoAbs that have multiple arginines in the region as well as 2C10 IgG have cell-penetrating properties (Foster et al. 1994; Song et al. 2008; Im et al. 2015). Cell-penetrating autoAbs might bind to the cell membrane, before being internalized and endocytosed into cells, via charge-charge interactions of positively charged amino acids in the CDR3s, with negatively charged sulfated polysaccharides or glycosaminoglycans, such as heparan sulfate, on the cell membrane (Faaber et al. 1986; Song et al. 2008; Im et al. 2015). The observations that 2C10 variable light domain (VL), which has no arginine residues in the CDR3 region, and G5-8 IgG, which has only one arginine in CDR3-VH, did not bind to antigen and did not penetrate into MES cells (Jang and Stollar 1990; Jang et al. 1998; Im et al. 2015) also provide evidence for the critical role of multiple arginines in 2C10 IgG and VH domains in cell-penetration.
By treating cells with a range of concentrations of 2C10 IgG and VH domain we showed here the reduction in cell survival and delay in cell cycle progress. It was also shown that G5-8 IgG, which does not penetrate into cells, did not affect cell cycle or survival rate. Results of a few earlier studies are in line with our present observations; cell death induced by penetrating anti-DNA Abs was previously demonstrated as a cytopathic effect in SLE and

![Figure 4](image-url)
LN (Graninger et al. 2000; Putterman 2004); human and murine anti-dsDNA autoAbs were cytotoxic to renal MES and tubular cells (Koren et al. 1995; Yu et al. 1998); anti-dsDNA Abs could induce cleavage of DNA and apoptosis (Kubota et al. 1996; Lee et al. 2007).

We clearly showed that 2C10 IgG and VH activated ERK1/2, p38 and RSK-1, and the activated RSK-1 was blocked by the p38 inhibitor, not by the ERK1/2 inhibitors. Similarly, there have been reports showing that RSK-1 was activated by p38 rather than by ERK1/2 in kidney epithelial or dendritic cells (Zaru et al. 2007, 2015; Luo et al. 2017). In 2C10 IgG and VH treated MES cells, p38-dependent MAPKAPK-2 activation and MAPKAPK-2-dependent RSK-1 activation were induced. Regulation of RSK phosphorylation by p38 through the activation of MAPKAPK-2 we observed is consistent with previous observations (Zaru et al. 2007, 2015; Anjum and Blenis 2008). It has been known that RSK-1 may induce inflammatory cytokines by a nuclear factor kappa beta (NF-κB)-independent pathway or triggering cyclooxygenase-2 through the activation of cAMP response element binding protein (Wu 2006; Moens et al. 2013). At least, possible involvement of NF-κB in our system could be also excluded, because it was shown that 2C10 did not activate NF-κB in MES cells (Im et al. 2015). It was previously reported that p38-MAPKAPK-2 induced prostaglandin-2 leads to cyclooxygenase-2 activation and gene expression of TNF-α, IL-6 and IL-1β (Bhatia et al. 2017). It was also reported that stronger p38 activation is promoted by TLR4 and TLR5 ligands versus stronger ERK1/2 activation by TLR2 (Zaru et al. 2007, 2015). Previous reports have demonstrated the association of TLRs and anti-dsDNA Abs in in vitro and in vivo animal experiments, including transgenic mice (Qing et al. 2008; Lee et al. 2010, 2014); nephro- 

genic anti-dsDNA Abs upregulate pro-inflammatory gene expression, such as high mobility group protein B1 (HMGB1) and TLR 2/4, in MES cells, and HMGB1 might be a pro-inflammatory mediator in Ab-induced kidney injury in SLE. Further study of involvement of TLRs, HMGB1 and prostaglandin-2 in our system is currently under investigation, and preliminary results indicate that HMGB1 level is increased at the protein level by cell-penetrating Abs including 2C10. Production of pro-inflammatory cytokines is a major factor involved in the pathogenesis of various diseases. The defined p38-MAPKAPK2-RSK1 pathway, activated by 2C10 in MES cells, may have an important role in stimulation of TNFα, IL-6 and IL-1β. Furthermore, p38 activated ATF-2 in 2C10 treated MES cells. Important targets of MAPK signaling are the activator protein-1 (AP-1) transcription factors, to which ATF-2 belongs. AP-1 complexes regulate cell-cycle progression and cell survival in a cell-type-specific manner although the molecular mechanisms are still poorly understood. However, AP-1 target genes are known to be pro-inflammatory cytokines (Qiao et al. 2016), and have been implicated in apoptosis in response to stress factors (Jacobs-Helber et al. 1998). Possible relationships between the activated ATF-2 and cytokine stimulation as well as reduction of cell proliferation shown in our system should be pursued further. When we treated 2C10 with a p38 inhibitor in MES cells, stimulation of the pro-inflammatory cytokine transcription was blocked, indicating that 2C10 IgG and VH stimulated the pro-inflammatory cytokines under the regulation of p38 MAPK signaling pathway in MES cells.

2C10 IgG and VH upregulated Bcl-2 in MES cells, suggesting Bcl-2 is also related to LN pathogenesis. The upregulated Bcl-2 levels were suppressed by RSK inhibitor, not ERK1/2 inhibitor, indicating that RSK is the upstream regulator of Bcl-2 in 2C10 treated MES cells. Predominant expression of RSK in kidney cells and regulation of Bcl-2 family proteins by RSK1 and 2 has been demonstrated previously (Bonni et al. 1999; Shimamura et al. 2000; Anjum and Blenis 2008).

We suggest that a subset of anti-dsDNA autoAbs such as positively charged CDR3-VH-containing Abs collectively could play a pathogenic role in SLE and LN through a mechanism of MES cell-penetration, resulting in the stimulation of pro-inflammatory cytokines through p38-MAPKAPK-2-RSK-1 and ATF-2 signaling pathways, as well as affecting the cell cycle and cell survival. Bcl-2 might also play a role in the pathogenic function of the Ab.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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