Prednisolone Sodium Succinate Down-Regulates BSAP/Pax5 and Causes a Growth Arrest in the Nalm6 Pre-B Cell Line

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RAHMAN, M., HIRABAYASHI, Y., ISHII, T., WATANABE, M., LIU, M. and SASAKI, T. *Prednisolone Sodium Succinate Down-Regulates BSAP/Pax5 and Causes a Growth Arrest in the Nalm6 Pre-B Cell Line.* Tohoku J. Exp. Med., 2001, **193**(3), 237-244 — Glucocorticoids (GCs) induce growth arrest and apoptosis of B lineage cells, especially pre-B and immature B cells, but little is known about this mechanism. Since B cell-specific activator protein (BSAP) encoded by the Pax5 gene is an essential transcription factor for B-cell proliferation, we have investigated the effect of prednisolone sodium succinate (Pss) on BSAP expression in several human leukemic B lineage cell lines. Pss caused growth arrest of Nalm6 (pre-B) cells, but this is not the case for 207 (pro-B) and Raji (mature B) cells. In Nalm6 cells, the growth rate and the amount of BSAP bound to its recognition sequence decreased in proportion to the Pss dose. Pss also reduced the expression level of BSAP as well as of Pax5 mRNA. These data suggest that the suppression of Pax5/BSAP may be involved in the GCs-induced growth arrest of pre-B cells.

——— glucocorticoids; BSAP; Pax5; growth arrest

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Glucocorticoids (GCs) have been widely used as effective therapeutic agents for autoimmune diseases and lymphoid malignancies because GCs induce growth arrest and apoptosis in B as well as T lymphocytes (Andréau et al. 1998). GCs induce significant degrees of apoptosis in normal murine B220⁺ and IgM⁺ marrow cells at physiological concentrations (Garvy et al. 1993). Moreover, the susceptibil-

ity to GCs-induced apoptosis is stage-dependent in developing B-cells. IgM⁺IgD⁻ (immature B) cells and B220^{du11}IgM⁻ (pre-B) cells are more susceptible than IgM⁺IgD⁺ (mature B) cells and B220^{du11} CD43⁺ (pro-B) cells (Merino et al. 1994). Little is known, however, about the molecular mechanism of GC-induced growth arrest and apoptosis in the B lineage cells. To clarify this point, we focused on the effect of

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GCs on the expression of the B cell-specific activator protein (BSAP), which is the key regulator of B-cell differentiation and proliferation.

BSAP encoded by the Pax5 gene is a DNA binding transcription factor, and regulates several important molecules for B cells (Busslinger and Nutt 1998; Nutt et al. 1998). The BSAP expression starts at the pro-B cell stage and terminates at the plasma cell stage. BSAP is up-regulated by stimulatory signals, and the suppression of BSAP by antisense oligo DNA reduced the proliferation of B cells, indicating that BSAP positively regulates B cell proliferation (Wakatsuki et al. 1994).

The present study was performed to investigate the effect of GCs on the expression of BSAP in human leukemic B-cell lines at various maturation stages. Prednisolone sodium succinate (Pss) was adopted for this study because it has been mainly used in practical therapies among GCs. Here we report that Pss-induced growth arrest occurred concurrently with the down-regulation of BSAP in Nalm6 cells.

MATERIALS AND METHODS

Cell lines and cell culture

The human cell lines that were used are as follows: Raji (mature B), Nalm6 (pre-B), Reh (pro-B), Nalm16 (pro-B), and 207 (pro-B). All of the cell lines were grown at 37°C in a humidified atmosphere at 5% CO₂ in RPMI1640 medium supplemented with 10% fetal calf serum, 2 mmol/liter L-glutamine, 50 mg/ml streptomycin, and 50 U/ml penicillin. Exponentially growing cells were used throughout all of the experiments at a concentration of 1 × 10⁵ to 5 × 10⁵/ml. Cell number and cell viability were assessed by trypan blue exclusion. The cell viability at the beginning of each experiment was greater than 95%.

Electrophoretic mobility shift assay

The double-stranded oligonucleotide probe, 5'-GCAGACACCCATGGTTGAGTGCCCTC-CAGG-3' (The underlined nucleotides represent the BSAP recognition sequence in the human CD19 promoter), was used for Electrophoretic mobility shift assay (Kozmik et al. 1992). For direct binding assays, the 5'-end of the sense-strand of the probe was labeled with fluorescein (FITC). The binding reaction mixture (20 µl final volume) contained the probe in the binding buffer (20 mM HEPES pH 7.6, 30 mmol/liter KCl, 10 mmol/liter (NH₄)₂SO₄, 1 mmol/liter DTT), 1 mM ethylene-diaminetetraacetic acid (EDTA), 0.2% Tween 20 and 3 µg poly(dI)-poly (dC). Nuclear extracts were prepared as described elsewhere (Schreiber et al. 1989). Approximately 10 µg of the extracts was added to each reaction mixture. The reaction mixtures were incubated for 30 minutes at room temperature and were subsequently electrophoresed in 6% polyacrylamide gel in 0.25 × TBE buffer (1 × TBE: 89 mM Tris-HCl, 89 mM Boric acid, 2 mM EDTA). The gels were scanned by FMBioII (Hitachi, Hitachi).

Western blot analysis

About 10 µg of the nuclear extracts was loaded into each well, separated on a 10% sodium dodecyl sulfate-polyacrylamide gel, and electrophoretically transferred to a polyvinylidene fluoride membrane (NEN Life Science Product, Boston, MA, USA). After blocking in TBS-T (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween20, pH 7.4) containing 5% nonfat dry milk for 1 hour at room temperature, the membrane was incubated with a 1/1000 dilution of goat polyclonal anti-BSAP (C-20) antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) in TBS-T for 1 hour. The membrane was then washed three times with TBS-T and incubated with a 1/10 000 dilution of alkaline phosphatase-conjugated anti-goat antibody

(Sigma, St. Louis, MO, USA) for 1 hour. After washing twice with TBS-T, the blot was developed with a 5-bromo-4-chloro-3-indolyl phosphate substrate (Sigma).

Real-time quantitative RT-PCR

The total RNA was isolated from the Nalm6 cells, which had been cultured in the absence or presence of Pss, using Isogen-LS™ (Nippon Gene, Tokyo) as per the manufacturer's protocol. RNA samples were further purified by DNase digestion and extraction with phenol and chloroform. The amount of the Pax5 mRNA was measured by the real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) method using the TaqMan™ RT-PCR kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA) and the ABI PRISM™ 7700 Sequence Detection Systems (Perkin Elmer Applied Biosystems). The total RNA was dissolved in a 50 μ l amplification reaction solution containing 1 $\times$ TaqMan buffer, 5.5 mM MgCl₂, 200 μ M of each dATP, dCTP, dGTP, and dUTP, 0.25 units AmpliTaq™ DNA polymerase, 0.02 units Reverse transcriptase, 200 nM of each primer, and 66 nM of TaqMan probe. The primers and TaqMan™ probe for the Pax5 gene were designed by using the Primer Express software program (Perkin Elmer Applied Biosystems). The primers for the D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene included in the TaqMan™ Rodent GAPDH Control Reagent (Perkin Elmer Applied Biosystems) were used. Primers and probes used in TaqMan™ PCR are as follows: Pax5 forward primer 5'-GCGAGAAGCTCTTTAGTTCCTTAATC-3', Pax5 reverse primer 5'-CTGATCACTGAGCTGAAACTAAACG-3', Pax5 TaqMan probe 5'-TTCACGGTGCCTTCGGACGCTT-3', GAPDH forward primer 5'-GAAGGTGAAGGTCGGAGTC-3', GAPDH reverse primer 5'-GAAGATGGTGGTGGGATTTTC-3'. The 5' end and the 3' end of TaqMan™ probes were

labeled with fluorescent carboxyfluorescein (FAM) (reporter) and fluorescent Tetra methyl-rhodamine (TAMRA) (quencher), respectively. PCR amplification of the complementary DNAs was performed using 1 μ l of the total RNA under the following conditions: first cycle consisting of 50°C for 60 minutes (RT reaction) and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minutes. The size of the amplified product was 87 bp for Pax5 transcript and 226 bp for GAPDH transcript. During PCR amplification, 5' nucleolytic activity of Taq polymerase cleaves the probe separating the 5' reporter fluorescent dye from the 3' quencher dye. Threshold cycle, Ct, which correlates inversely with the target mRNA levels, was defined as the fractional cycle number at which the reporter fluorescent emission increases above a threshold level. Δ Ct was determined by subtraction of the Ct of GAPDH from that of Pax5 in each sample, and then, $\Delta\Delta$ Ct was determined by subtraction of the Δ Ct of Pax5 with Pss from that of Pax5 without Pss. The fold difference was indicated by $2^{-\Delta\Delta$ Ct and the relative amount of Pax5 mRNA in the Pss treated cells versus the control cells was calculated by $1/2^{-\Delta\Delta$ Ct} \times 100 (%).

RESULTS

Pss inhibits proliferation of Nalm6 pre-B cells

To investigate the effect of GCs on B-lymphocytes, several leukemic B cell lines of different maturation stages were treated with Pss. Fig. 1A shows the time course studies of the growth of Raji (B), Nalm 6 (pre B), and 207 (pro B) cells which were cultured in the absence or presence of 1 μ M Pss (Fig. 1A). The growth rate of Pss-treated Nalm6 cells began to decrease after 24 hours. The viability of Pss-treated Nalm6 cells began to decrease after 48 hours, whereas it had been more than 95% within 48 hours. On the other hand, no significant changes were observed in the growth and viability of Pss-treated Raji (mature B)

Fig. 1. Effect of Pss on the proliferation of human leukemic B lineage cells. (A) Exponentially growing cells were cultured in the absence (control) or presence of 1 μ M Pss for 4 days. The mean of three independent experiments is plotted. ○, Nalm6 Pss(-); ●, Nalm6 Pss(+); △, 207 Pss(-); ▲, 207 Pss(+); □, Raji Pss(-); ■, Raji Pss(+). (B) Nalm6 cells were cultured for 36 hours in the absence (control) or presence of Pss at the indicated concentrations. Each point represents the mean $\pm$ s.d. of values obtained from triplicate samples.

and 207 (pro-B) cells within 4 days. Also, no significant changes were observed in Pss-treated Reh (pro-B) and Nalm16 (pro-B) cells (data not shown). Then, Nalm6 cells were cultured for 36 hours in the absence or presence of Pss at the indicated concentrations (Fig. 1B). Pss inhib-

ited the proliferation of Nalm6 cells in a dose dependent manner.

Pss decreased the amount of BSAP bound to its recognition sequence in Nalm6 cells

To test whether Pss reduces the amount of BSAP bound to its recognition sequence, electrophoretic mobility shift assay (EMSA) was employed. Cells were cultured for 36 hours in the absence (control) or presence of Pss at the indicated concentrations prior to harvesting. The amount of BSAP bound to the probe decreased in proportion to the Pss dose in Nalm6 cells while cell viability was not affected and was more than 95% in all of the experiments (Fig. 2A). This is not the case for 207 and Raji cells (Figs. 2B and 2C). Also, Nalm6 cells were cultured for 6, 12, and 24 hours in the absence (control) or presence of 1 mM or 100 nM Pss prior to harvesting, and similar results were obtained (Fig. 2D). The amount of BSAP already decreased at 6 hours in the presence of Pss. Anti-BSAP antibody caused a supershift of the detected band, indicating that the band contains BSAP (Fig. 2E).

Pss down-regulates BSAP expression in Nalm6 cells

The level of BSAP in the nuclear extracts from Nalm6 cells was evaluated by western blot analysis. Nalm6 cells were cultured for 24 hours in the absence (control) or presence of Pss prior to harvesting. Similar to the results on EMSA, the intensity of BSAP expression decreased in proportion to the Pss dose (Fig. 3A). Non-specific proteins were stained with Coomassie blue, and there was no difference in the intensity of the bands (Fig. 3B).

Then, to test whether Pss decreases the amount of Pax5 mRNA in Nalm6 cells, it was measured by the real time quantitative RT-PCR method. Nalm6 Cells were cultured for the indicated times in the absence (control) or

Fig. 2. Effect of Pss on the amount of BSAP bound to its recognition sequence. (A) Nalm6, (B) 207, and (C) Raji cells were cultured for 36 hours in the absence (control) or presence of Pss at the indicated concentrations prior to harvesting. x: Cells were cultured without Pss, and a 100-fold molar excess of unlabeled probe was added. y: only FITC labeled probe but no nuclear extracts was added. (D) Nalm6 cells were cultured for 6, 12, and 24 hours in the absence (control) or presence of Pss at the indicated concentrations prior to harvesting. These data are representative of results obtained in 3 independent experiments. (E) The band detected contains BSAP. The nuclear extracts from Nalm6 cells were used. 1: FITC labeled probe only. 2: FITC labeled probe with a 100-fold molar excess of unlabeled probe. 3: FITC labeled probe with 2 μ g of anti-BSAP (C-20) antibody.

presence of Pss before harvesting for total RNA isolation. The amount of the PAX5 mRNA in Pss-treated cells already decreased at 3 hours and remained constant after 6 hours (Fig. 4).

DISCUSSION

In this report, we show that Pss decreased the growth rate of Nalm6 cells, amount of BSAP bound to its recognition sequence, and the expression level of BSAP in a dose dependent manner. We would like to point out the following possibilities should to assess the

results. Firstly, growth arrest of Nalm6 cells and down-regulation of BSAP occurred simultaneously but independently. Secondly, the down-regulation of BSAP caused growth arrest of Nalm6 cells. Thirdly, Pss down-regulates not only BSAP but also other important molecules for growth. Fourthly, growth arrest causes the down-regulation of BSAP. However, since the suppression of BSAP by antisense oligo DNA reduced the proliferation of B cells, the second possibility is most likely to participate in growth arrest of Nalm6 cells. If Pss down-regulates other important molecules

Fig. 3. Pss down-regulated BSAP expression in Nalm6 cells. Nalm6 cells were cultured for 36 hours in the absence (control) or presence of Pss at the indicated concentrations prior to harvesting. (A) Western blot analysis was performed to show the level of BSAP expression. (B) Coomassie blue staining was employed to show the amount of non-specific proteins.

for growth, the growth of other cells may also be inhibited.

Since the level of Pax5 mRNA decreased in the Pss-treated cells, Pss may down-regulate Pax5 transcription or decrease the Pax5 mRNA stability. GCs bind to the glucocorticoid receptor, and then they bind to the glucocorticoid responsive element (GRE) in the primary response gene. In some cases, the product of the primary response gene regulates secondary response genes (Dean and Sanders 1996). We searched GRE in the 5'-flanking region of the human PAX-5 gene, but no GRE was found within about 2.4 kb upstream sequence from the start codon of exon 1A and about 7 kb upstream of exon 1B (unpublished data). This suggests that the Pax5 gene may be a secondary response gene if the Pax5 mRNA stability is not affected.

Currently, six target genes of BSAP (CD19, mb-1, PD-1, *n-myc*, LEF-1, and bcl-xL) have been identified by loss- and gain-of function experiments (Nutt et al. 1998). Ig- α , which is

Fig. 4. Pss decreased the level of Pax5 mRNA in Nalm6 cells. Nalm6 cells were cultured with 1 μM Pss for 3, 6, 9, 12, and 24 hours prior to harvesting. The ratio of the Pss-treated cells vs. the control cells at each time point is shown in percentage. Each point represents the mean $\pm$ s.d. of values obtained from triplicate samples.

encoded by the mb-1 gene and up-regulated by BSAP, forms a complex with Ig- β , and the complex mediates signal transduction through the pre-B and B-cell receptors. The signals through these receptors are essential for proliferation. A 10-fold reduction in Ig- α expression was observed in Pax5 deficient mice. PD-1 is a transmembrane protein and negatively regulates B-cell proliferation (Nishimura et al. 1998). The increased sensitivity to antigenic stimulation was observed in mice lacking PD-1. PD-1 expression was increased in Pax5 $^{-/-}$ pro-B (pre-BI) cells. LEF-1 is a nuclear transcription factor and only expressed in pro-B and pre-B cells in the B lymphoid lineage (Travis et al. 1991). Mice lacking LEF-1 exhibited defects in pro-B cell proliferation and survival (Reya et al. 2000). LEF-1 expression was decreased in Pax5 $^{-/-}$ pro-B (pre-BI) cells (Nutt et al. 1998). Bcl-xL, which is an anti-apoptotic factor, is mainly expressed in pre-B cells in the B lymphoid lineage. Bcl-xL expression was also decreased in Pax5 $^{-/-}$ pro-B (pre-BI) cells (Nutt et al. 1998). These data raise the possibility that suppression of BSAP by Pss may

up-regulate PD-1 and down-regulate Ig- α , LEF-1, and bcl-xL and consequently inhibit cell proliferation and survival. CD19, which is exclusively regulated by BSAP, acts as a co-stimulatory molecule to lower the threshold for antigen-dependent signaling (Tedder et al. 1997). However, CD19 may not be involved in steroid induced growth arrest of pre-B cells because the B-cell development in the bone marrow of CD19 $^{-/-}$ mice was normal. *N-myc* is mainly expressed in pre-B cells in the B lymphoid lineage (Smith et al. 1992). *N-myc* expression was decreased in Pax5 $^{-/-}$ pro-B (pre-BI) cells (Nutt et al. 1998). However, the role of *N-myc* is not yet clearly determined because *N-myc* is involved in apoptosis as well as in proliferation.

GCs are useful therapeutic agents for autoimmune diseases. The ultimate aim of therapy is clonal deletion of autoreactive cells. In the case of B-cells, B-cell receptor, which determines the antigen specificity, is formed at the pre-B cell stage and expressed on the cell surface at the immature B cell stage. Therefore, elimination of the pre-B and immature B cells expressing autoreactive B-cell receptor means the clonal deletion of the autoreactive B lineage cells. Bcl-xL is crucial for early B-cell development and survival. The analysis of Bcl-xL transgenic mice demonstrated that Bcl-xL can protect pre-B and immature-B cells against GCs-induced apoptosis (Grillot et al. 1996). Overexpression of Bcl-xL in B-lymphoid lineage cells induced self-reactive B cells (Fang et al. 1998). These data suggest that the level of Bcl-xL may be important to eliminate autoreactive B cell clones. Clinically, high-dose steroid pulse therapy has been employed to suppress the activities of autoimmune diseases. Plasma corticosteroid levels after intravenous administration of 1 g of methylprednisolone is increased up to about 50 mg/liter (134 μ M) and decreased with a half time of 3 hours. After 24 hours, the level is still maintained around 200 μ g/liter (540 nM). If

the sensitivity against steroids of pre-B cells in vivo is similar to the one of Nalm6 cells, and if the reduction of BSAP by GCs decreases the level of Bcl-xL, this therapy not only inhibits the proliferation of pre-B cell but also makes the cells become sensitive to apoptosis. Attempts to clarify the precise mechanism of suppression in BSAP by GCs and the targets of BSAP in pre-B cells are currently in progress.

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