Abstract. Background: Primary effusion lymphoma (PEL) is a rare but aggressive form of non-Hodgkin's B-cell lymphoma in immunodeficient patients. Resistance to conventional chemotherapeutic regimens is common in PEL and contributes to a very poor prognosis; hence, novel potent anti-PEL agents are required. Anticancer effects of non-steroidal anti-inflammatory drugs (NSAIDs) are well-established in epithelial cancer but are unclear in hematological malignancies. Therefore, the anticancer activities of selected NSAIDs, sodium salicylate (NaS), on PEL cell lines are of interest. Materials and Methods: Anti-proliferation of NaS on PEL cell lines was shown by MTT. Apoptosis induction and caspase activations were determined by flow cytometry analysis. ROS production was accessed by DCFH-DA. Western blot was performed to determine molecular mechanisms. Results: NaS effectively inhibited cell proliferation of all PEL cell lines. Caspase-dependent apoptosis was demonstrated and simultaneous induction of reactive oxygen species production and c-Jun N-terminal kinases (JNK)-p38 activation was observed prior to apoptosis induction, and these might be responsible for NaS-induced apoptosis. Conclusion: Significant anticancer effects of NaS on PEL cell lines were found. A novel role of NaS for PEL treatment is suggested.
Anticancer activity of NaS is anti-proliferative and mainly through induction of apoptosis. Major molecular pathways targeted by NaS are mitogen-activating protein kinase (MAPK) and nuclear factor-κB (NF-κB) pathways. NaS induced p38 activation in a colon cancer cell line (9), while it inhibited NF-κB activity in prostate cancer (10). Moreover, p38-dependent NF-κB inhibitory effect of NaS was demonstrated in a non-cancer cell line (14). NaS-activated reactive oxygen species (ROS)-dependent apoptosis was also evident in epithelial cancer (15). These mechanisms are mainly found in epithelial cancer. Little is known regarding the molecular mechanism of NaS effects on hematological malignancies. Therefore, we focused on the molecular mechanism of NaS-induced on PEL cell death and apoptosis in the present study.

Materials and Methods

Cell lines and reagents. The human PEL cell lines, which acquired HHV-8 infection without EBV co-infection including BCBL-1 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIH, Bethesda, MD, USA) (16), BC-3 (American Type Culture Collection, Manassas, VA, USA) (17), GTO (18) and TY-1 (19) were maintained in RPMI-1640 (American Type Culture Collection, Manassas, VA, USA) (17), supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified incubator at 37˚C with 5% CO2. NaS was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Tetrazolium dye MTT assay. The antiproliferative effects of NaS against PEL cell lines were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. In brief, 2×104 cells per well were seeded in triplicate into a 96-well plate and then different concentrations of NaS were added. Cells were treated for 24, and 48 h. Subsequently, MTT (Sigma-Aldrich) was added to obtain a final concentration of 0.5 mg/ml. Formazan crystals were then dissolved by 0.01 N HCl in isopropanol and OD595 was obtained a final concentration of 0.5 mg/ml. Formazan crystals were then dissolved by 0.01 N HCl in isopropanol and OD595 was determined by a microplate reader (Multiskan; Thermo Electron, Waltham, MA, USA). OD595 of NaS treated samples was normalized by samples without NaS at each time point.

Sub-G1 population analysis. After NaS treatment for 24 h, cells were washed with cold phosphate buffered saline (PBS) and fixed with 70% ethanol at 4˚C overnight. Fixed cells were then washed with cold PBS and stained with propidium iodide (PI; 10 μg/ml in PBS) for 30 min in the dark. Samples were analyzed by flow cytometry (LSRII; BD Bioscience, San Jose, CA, USA) and data were analyzed on FlowJo software (Tree Star Inc., Ashland, OR, USA).

Annexin V binding assay. Apoptosis was quantified using an Annexin V-FITC Apoptosis Detection Kit (eBioSciences, San Diego, CA, USA) as recommended by the manufacturer. Briefly, after treatment with NaS for 12, and 24 h, cells were stained with annexin V-FITC and PI before analysis by flow cytometry.

Caspase-3, -8, and -9 activities. The activities of active caspase-3, -8, and -9 were measured by APOPCYTO intracellular caspase-3, -8, -9 activity detection kits (MBL, Nagoya, Japan) according to the manufacturer’s instructions. NaS-treated and -untreated cells were incubated with 10 μM specific caspase substrates (FITC-DEVD-FMK for caspase-3, FITC-LEHD-FMK for caspase-9, and FITC-IETD-FMK for caspase-8) for 60 min at 37˚C, washed with 1 μg/ml of PI in washing buffer and analyzed by flow cytometry. Cells with active caspase among PI-negative (living) cells were demonstrated.

Protein extraction and western blot analysis. To prepare total protein lysate, BCBL-1, and TY-1 cells with or without treatment of NaS were collected at different times and washed in cold PBS before the addition of lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM NaF, 1 mM Na3VO4, and protease inhibitor cocktail. Cells were incubated on ice for 30 min, centrifuged at 15,000 xg for 15 min, and the supernatant was collected. Protein amounts were determined by the bicinchoninic acid (BCA) protein assay (Thermo Scientific, Rockford, IL, USA). Protein extracts were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then blotted onto a polyvinylidine difluoride (PVDF) membrane (GE Healthcare, Tokyo, Japan). Detection was performed using Chemilumino One Super reagents (Nacalai Tesque, Tokyo, Japan).

Sub-0.1% population analysis. After NaS treatment for 24 h, cells were washed with cold phosphate buffered saline (PBS) and fixed with 70% ethanol at 4˚C overnight. Fixed cells were then washed with cold PBS and stained with propidium iodide (PI; 10 μg/ml in PBS) for 30 min in the dark. Samples were analyzed by flow cytometry (LSRII; BD Bioscience, San Jose, CA, USA) and data were analyzed on FlowJo software (Tree Star Inc., Ashland, OR, USA).

Measurement of ROS production. Production of ROS was determined by fluorescent probe 2’-7’-dichlorodihydrofluorescein diacetate (DCFH-DA; Sekisui Medical, Tokyo, Japan). Cells were incubated with 20 μM DCFH-DA for 30 min, treated with 8 mM NaS for 5 min to 3 h, and immediately analyzed by the flow cytometry. Fold changes of mean fluorescent intensities (MFI) at specific times were calculated by the following formula:

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\text{Fold change} = \frac{\text{MFI of NaS treated cells}}{\text{MFI of untreated control cells}}
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For dose-dependent analysis, cells were treated by 0, 4, or 8 mM NaS for 1 h and were incubated with 10 μM DCFH-DA for 30 min before analysis with flow cytometry.

Statistical analysis. The statistical significance of the differences observed between experimental groups was determined using the Student’s t-test. A value of p<0.05 was considered significant.

Results

Antiproliferative effects of NaS on PEL. Effects of NaS on proliferation of HHV-8 harboring PEL cells were determined by the MTT assay. The results showed that NaS effectively inhibited PEL cell proliferation. At 24 h, IC50 of NaS against BCBL-1, BC3, GTO and TY-1 were 6.49, 3.55, 5.34, 5.37 mM respectively.
and IC50 at 48 h were 4.32, 2.38, 2.68, 4.16 mM, respectively. Dose- and time-dependent anti-proliferative effects of NaS against PEL cell lines are demonstrated in Figure 1.

NaS Induces caspase-dependent apoptotic cell death. NaS-induced apoptosis is known in both epithelial and hematological malignancies (9, 11). We characterized the effects of NaS on apoptosis induction in PEL cell lines BCBL-1 and TY-1. Early apoptotic events characterized by phosphotidylserine (PS) exposure and late apoptosis characterized by sub-G1 populations were determined. For exposed PS determination, annexin V binding assay was performed. Cells were treated with 0, 4 or 8 mM NaS for 24 h and were then analyzed. The results show that NaS induced PS exposure (Figure 2A). Concomitantly, the sub-G1 population detected by PI staining was also increased (Figure 2B) in NaS-treated cells. NaS-induced apoptosis occurred in a dose- and time-dependent manner (Figure 2C).

The mechanism of apoptosis was further characterized. Cells were treated with 8 mM NaS for 12 and 24 h and then the activities of intrinsic caspase-9, extrinsic caspase-8, and common caspase-3 were measured by determination of specific caspase substrate cleavage. The results showed increased caspase-3, -8, and -9 activities in NaS-treated cells in a time-dependent manner (Figure 2D). Consistent results were obtained when proteolytic activations of caspases were determined by western blot (Figure 2E). Increased active cleaved isoforms of caspase-3, -8, and -9 were observed in BCBL-1 and TY-1 cells treated with 8 mM NaS when compared to untreated cells. Activation of caspases was detected as early as 3 to 6 h after NaS treatment and persisted until 24 h. Altogether, NaS-induced apoptosis in PEL cell lines was caspase-dependent.

NaS-activated ROS production. NaS-induced ROS production and then apoptosis was demonstrated in epithelial cancer cell lines (15, 20). To determine the underlying mechanisms by which NaS induced apoptosis in PEL cell lines, BCBL-1 and TY-1 cells were treated with 4 mM or 8 mM NaS for 1 h and the accumulation of ROS was determined by DCFH-DA assay. The results showed increased production of ROS in both PEL cell lines in a dose-dependent manner (Figure 3A).

To further characterize ROS generation in PEL cell lines, BCBL-1 and TY-1 cells were treated with 8 mM NaS and ROS was determined from 5 min to 3 h after NaS addition. The result demonstrated in Figure 3B show that increased ROS production was observed as early as 5 min after NaS treatment and the accumulation of ROS occurred in a time-dependent manner throughout the observations. These data show ROS induction by NaS in BCBL-1 and TY-1 cells.

NaS induced JNK-p38 activation. The relationships between ROS production, JNK, p38 and ERK pathways and apoptosis induction are established (21, 22). To show the effects of NaS on MAPK pathways in PEL cell lines. BCBL-1 and TY-1 were treated with 8 mM NaS and cells were collected at 5, 10, 30 min, 1, 3, and 6 h. Cell lysates were prepared and
signaling molecules in MAPK pathways were analyzed by western blot analysis. The results show increased JNK and p38 phosphorylation in BCBL-1 and TY-1 cells at 5 min and 3 h after NaS treatment. No ERK activation was observed in NaS-treated cells at any time points. Collectively, these results indicate that the effect of NaS on PEL cell line appears to be mediated via JNK-p38.

Discussion

The effects of NaS, the active metabolite of aspirin, on cancer chemoprevention and cancer treatment are well-established (6). Little is known regarding the effects of NaS on an aggressive form of NHL, PEL. Therefore, the effects of NaS on PEL are of interest. The current study demonstrated that NaS effectively...
inhibits PEL cell proliferation in both dose- and time-dependent manners. The IC$_{50}$ of NaS against PEL is at a pharmacologically achievable dose and comparable to that for cell lines of other hematological malignancies. NaS effectively inhibited cell proliferation and induced apoptotic cell death. Apoptosis induction demonstrated by PS exposure, caspase-3, -8, and -9 activation, and sub-G$_1$ population, were shown in both BCBL-1 and TY-1 cell lines. The major mechanism of NaS-induced apoptosis is proposed to occur through ROS and JNK-p38 pathways.

Anti-proliferative activity of NaS against PEL cells was demonstrated in the current study. To avoid the combination effect of NaS on EBV expression which might effect PEL cell proliferation (23), PEL cell lines BCBL-1, BC3, GTO, and TY-1, which acquire human HHV8 but not EBV infections, were selected (18, 19, 24). The results showed that the IC$_{50}$ of NaS against all PEL cell lines ranged from 2-6 mM, which is in a systemically achievable range (14, 25). This is equivalent to growth-inhibitory doses reported in myeloid leukemia (11), B-CLL (12) and CTCL (13) cell lines, and lower than doses toxic to peripheral blood mononuclear cells (12). It is interesting to note that due to the uncommon presentation of PEL, which is generally localized in the body cavities, this might be an exceptional opportunity to administer NaS directly into confined body cavities without serious side-effects (26).

The induction of apoptosis demonstrated by several apoptotic hallmarks in the present study are in agreement with other reports that NaS induces caspase-3 and -9 activation (12, 13, 27), PS exposure (11), and accumulation of sub-G$_1$ population (12, 13). It has been demonstrated that caspase activation is partly a consequence of mitochondrial transmembrane potential disruption (13, 15) and inhibition of expression of anti-apoptotic B-cell lymphoma-2 (Bcl-2) family members (11). Activation of extrinsic caspase-8 by NaS treatment is consistent with a previous report for the HL60 myeloid leukemia cell line (27). NaS brings about apoptotic cell death of PEL cell line in a caspase-dependent manner and both intrinsic and extrinsic pathways are activated.

Mechanisms of NaS-induced apoptosis have been reported previously. These are mainly through generation of oxidative stress and activation of MAPK signaling pathways (9, 14) and the inhibition of NF-$\kappa$B signaling pathway (10). NaS-potentiated ROS production has previously been reported in epithelial tumor cell lines, including colon cancer, gastric cancer, and glioma (15, 20) but not hematological malignancy. We demonstrated induction of ROS production by NaS in PEL cell lines. ROS accumulation occurred as early as 5 min after 8 mM NaS treatment. Concurrently, phosphorylation of JNK and p38 were observed. Activation of JNK by salicylate has been shown to be cell type-dependent (28). JNK activation is detected in NaS-treated COS-1 monkey kidney cells but not normal human fibroblasts. In Schwenger’s study, JNK activity was detected as early as 15 min after a high dose (20 mM) of NaS treatment (28), comparable to our results that JNK phosphorylation was observed at 5 min after 8 mM NaS treatment. A faster response to a lower dose of NaS observed in PEL cells might imply the increased susceptibility of JNK to activation in PEL cell lines. Increased p38 phosphorylation was also observed in HCT116 colon cancer cells and inhibition of p38 by specific inhibitor, SB203580, abrogated NaS induced apoptosis (9). NaS-induced apoptosis in PEL cell lines through ROS and JNK-p38 pathway is proposed.

Figure 3. NaS-activated reactive oxygen species (ROS) production. ROS accumulation in BCBL-1 and TY-1 cells was determined by DCFH-DA assay. A: Cells were treated with 0 (control), 4, and 8 mM NaS for 1 h, subsequently incubated with DCFH-DA for 30 min, and then analyzed by flow cytometry. B: DCFH-DA-pretreated cells were incubated with 8 mM NaS for the indicated times and then ROS production was determined. Fold changes were calculated by normalization of the mean fluorescent intensity (MFI) of NaS-treated cells with the MFI of untreated cells collected at the same time. Data are presented as the mean±SD from three independent experiments. *p<0.05, **p<0.001.
The effects of NaS on NF-κB signaling molecules are well-established on both tumor necrosis factor (TNF)-dependent (14, 29, 30) and other stimulation (10, 29). NF-κB inhibition is partly explained through p38-inhibited TNF-induced inhibitor of κB (IκB) phosphorylation (14). In the present study, neither the inhibition of IκB phosphorylation nor the induction of NF-κB nuclear translocation was observed (data not shown). This is consistent with a previous report that in BC3 PEL cell line that without TNF stimulation, treatment of NaS did not inhibit constitutive NF-κB activity (31). Taken together, NaS induced apoptosis in PEL cell lines may not associate with NF-κB inhibition.

In summary, the potential roles of NaS in reduction of proliferation and induction of apoptosis are demonstrated in PEL cell lines. These effects are proposed to be a consequence of ROS production and JNK-p38 activation. This study might provide the basis for a novel strategy of using NaS for PEL treatment. Further study in an animal model is required. Direct administration of NaS to the PEL-affected body cavity is suggested.

Competing Interests
The Authors declare no competing interests.

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Figure 4. NaS induced JNK-p38 activation. NaS treated BCBL-1 and TY-1 were collected at the indicated times. A: Total protein lysates were prepared and phosphorylated-JNK (p-JNK), JNK, phosphorylated-p38 (p-p38), p38, phosphorylated-ERK, and ERK were determined by western blot. B: Dose-dependent activation of JNK and p38 were observed in BCBL-1 and TY-1 cells treated with the indicated concentration of NaS at 30 min and 3 h. Representative data from three independent experiments are presented.
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