Sulforaphane Exhibits Cytotoxic Effects against Primary Effusion Lymphoma Cells by Suppressing p38MAPK and AKT Phosphorylation

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Primary effusion lymphoma (PEL) is a rare subtype of non-Hodgkin’s B-cell lymphoma and is caused by Kaposi’s sarcoma-associated herpesvirus (KSHV) in immunosuppressed patients. PEL is an aggressive lymphoma and is frequently resistant to conventional chemotherapies. Sulforaphane (SFN), a natural compound found in cruciferous vegetables and broccoli sprouts, modulates signaling pathways and epigenetic gene expression. However, the anti-proliferative effects of SFN on PEL cells and the underlying mechanisms have not been identified. Here, we found that SFN decreased the viability of KSHV-infected PEL cells compared to KSHV-uninfected B-lymphoma cells. The anti-proliferative effects of SFN on PEL cells were mediated by apoptosis with activating caspases. In addition, SFN inhibited the phosphorylation of p38 mitogen-activated protein kinase (p38MAPK) and AKT in PEL cells. We also showed that p38MAPK and AKT inhibitors reduced PEL cell growth. The constitutive and/or transient activation of p38MAPK and AKT signaling are necessary for the survival and proliferation of PEL cells. Our data and previous literature indicate that SFN represses the phosphorylation of p38MAPK and AKT, which results in PEL cell apoptosis. Moreover, we investigated whether MG132 or sangivamycin (Sangi) in combination with SFN potentiated the cytotoxic effects of SFN on PEL cells. Compared to treatment with SFN alone, the addition of MG132 or Sangi enhanced the cytotoxic activity of SFN in a synergistic manner. In conclusion, the anti-proliferative effects of SFN indicate its potential as a new substance for the treatment of PEL.

Key words sulforaphane; AKT; p38 mitogen-activated protein kinase; primary effusion lymphoma; apoptosis; Kaposi’s sarcoma-associated herpesvirus

INTRODUCTION

Primary effusion lymphoma (PEL) is a rare subtype of non-Hodgkin’s B-cell lymphoma and is caused by Kaposi’s sarcoma-associated herpesvirus (KSHV) in immunosuppressed patients. PEL is an aggressive lymphoma and is frequently resistant to conventional chemotherapies. Sulforaphane (SFN), a natural compound found in cruciferous vegetables and broccoli sprouts, modulates signaling pathways and epigenetic gene expression. However, the anti-proliferative effects of SFN on PEL cells and the underlying mechanisms have not been identified. Here, we found that SFN decreased the viability of KSHV-infected PEL cells compared to KSHV-uninfected B-lymphoma cells. The anti-proliferative effects of SFN on PEL cells were mediated by apoptosis with activating caspases. In addition, SFN inhibited the phosphorylation of p38 mitogen-activated protein kinase (p38MAPK) and AKT in PEL cells. We also showed that p38MAPK and AKT inhibitors reduced PEL cell growth. The constitutive and/or transient activation of p38MAPK and AKT signaling are necessary for the survival and proliferation of PEL cells. Our data and previous literature indicate that SFN represses the phosphorylation of p38MAPK and AKT, which results in PEL cell apoptosis. Moreover, we investigated whether MG132 or sangivamycin (Sangi) in combination with SFN potentiated the cytotoxic effects of SFN on PEL cells. Compared to treatment with SFN alone, the addition of MG132 or Sangi enhanced the cytotoxic activity of SFN in a synergistic manner. In conclusion, the anti-proliferative effects of SFN indicate its potential as a new substance for the treatment of PEL.

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MATERIALS AND METHODS

Agents, Cell Lines, and Cell Culture SFN (Cayman Chemical, MI, U.S.A.), SB203580 (Wako, Osaka, Japan), AKT inhibitor VIII (Merck, NJ, U.S.A.), U0126 (Merck), Sangivamycin (Merek), and MG132 (Peptide Institute, Osaka Japan) were dissolved in dimethyl sulfoxide (DMSO). KSHV-positive PEL cell lines (BC2, BC3, and HBL6) and KSHV-negative B-lymphoma cell lines (DG75, Ramos, IB4, Bjab, and Raji) were cultured in RPMI 1640 containing 10% fetal bovine serum. The optical density of each sample was measured at 450 nm with a spec-
trophotometer (Tecan M200; Kanagawa, Japan) and is expressed as a percentage (the absorbance of DMSO-treated cells was defined as 100%).

Western Blotting and Antibodies Cells were solubilized in sodium dodecyl sulfate (SDS) sample buffer containing 1% 2-ME, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM NaF and 0.5 mM β-glycerophosphate. The used primary antibodies were as follows: cleaved caspase-3 (#9661), cleaved caspase-7 (#9491), cleaved poly(ADP-ribose)polymerase (PARP) (#5625), PARP (#9532), Thr308-phospho-AKT (#13038) and AKT (#9272) (Cell Signaling Technology, MA, U.S.A.); Thr183/Tyr185-phospho-c-Jun N-terminal kinase (JNK)/SAPK (612540), JNK/SAPK (610627), Thr202/Tyr204-phospho-ERK1/2 (612359) and ERK 2 (610103) (BD Biosciences, NJ, U.S.A.); and β-Actin (sc-69879) (Santa Cruz, CA, U.S.A.).

Statistical Analysis The statistical significance between each group and the control was analyzed by one-way ANOVA followed by Dunnett’s test for multiple comparisons (Figs. 1A, E). Statistically significant data were analyzed with GraphPad prism 7.

RESULTS AND DISCUSSION

First, we investigated the cytotoxic effects of various SFN concentrations on KSHV-infected PEL cell lines (BC2, BC3, and HBL6) and KSHV-uninfected B-cell lymphomas (DG75, Ramos, IB4, Bjab, and Raji). SFN remarkably inhibited the proliferation of PEL cells and did not affect the growth of uninfected B-cells. The cytotoxic effects of SFN on KSHV-positive PEL cells (BC2, BC3, HBL6) and KSHV-uninfected cells (DG75, Ramos, IB4, Bjab, Raji) were evaluated by a cell viability assay. Cells were incubated with the indicated concentration of SFN for 24 h and subjected to the cell viability assay. The viabilities of vehicle (DMSO)-treated cells were defined as 100% relative cell survival. * p < 0.05, ** p < 0.01 and *** p < 0.001 indicate the statistical significance compared to vehicle (DMSO)-treated cells. ns, not significant. (B) SFN induces apoptosis in PEL cells via caspase-9, -3 and -7 activation. PEL cells (BC2, BC3) and uninfected Ramos cells were treated with 20 µM SFN for 4–24 h and cell lysates were subjected to Western blot analysis using antibodies against the cleaved caspases and cleaved PARP. NT, non-treated cells. (C, D) SFN suppresses the phosphorylation of p38MAPK and AKT. BC3 PEL cells and KSHV-uninfected Ramos cells were treated with 20 µM SFN for 4–24 h, and cell lysates were subjected to Western blotting with phospho-specific antibodies. (E) Cytotoxic effects of SB203580 (p38MAPK inhibitor) or AKT inhibitor VIII on PEL cells. BC3 PEL cells and KSHV-uninfected Ramos cells were treated with SB203580 or AKT inhibitor for 24 h and were subjected to the cell viability assay. The viabilities of vehicle (DMSO)-treated cells were defined as 100% relative cell survival. * p < 0.05, ** p < 0.01 and *** p < 0.001 indicate the statistical significance compared with vehicle-treated cells. ns, not significant.
suppresses the growth of PEL cells by triggering apoptosis, which is mediated by caspase-9 pathway.

Several signaling pathways are activated in PEL cells, which are required for PEL cell survival and growth.\(^5\)\(^-\)\(^10\) Therefore, we analyzed changes in these pathways upon SFN treatment. When BC3 PEL cells and uninfected Ramos cells were cultured with 20 \(\mu\)M SFN, the phosphorylation of p38MAPK and AKT in BC3 cells were decreased as compared to that in Ramos cells (Figs. 1C, D). Since activation of p38MAPK and AKT pathways are necessary for the survival and proliferation of PEL cells,\(^7\)\(^-\)\(^10\) we hypothesized that SFN exerted cytotoxic activity against PEL cells. Thus, we examined the effects of AKT inhibitor VIII and SB203580 (p38MAPK inhibitor) on BC3 cell proliferation. AKT inhibitor VIII decreased the viability of BC3 cells compared to Ramos cells (Fig. 1E). SB203580 also tended to reduce the viability of BC3. These suggest that SFN suppresses p38MAPK and AKT signaling, resulting in PEL cell apoptosis.

Previously, we reported that the proteasome inhibitor MG132 and the adenosine analog sangivamycin (Sangi) induced apoptosis in PEL cells by suppressing NF\(\kappa\)B and ERK signaling, respectively.\(^6\)\(^,\)\(^8\) It is well known that combined therapies for lymphoma are more effective than monotherapies. Therefore, we investigated whether treatment with a low concentration of SFN in combination with MG132 or Sangi (also at low levels) induced cytotoxicity against PEL cells. BC3 cells were treated with SFN alone (Figs. 2A, G), MG132 alone (Fig. 2B), Sangi alone (Fig. 2H), MG132 and SFN (Figs. 2C–E), or Sangi and SFN (Figs. 2I–K). When BC3 cells were treated with 1 \(\mu\)M SFN alone, the cell viability decreased by approximately 10%, compared to DMSO-treated cells. However, the combination of 1 \(\mu\)M SFN with MG132 (1 or 2 \(\mu\)M) or 1 \(\mu\)M SFN with Sangi (5 or 10 nM) significantly suppressed the viability of BC3 cells in a synergistic manner. Moreover, the effects of MG132 or Sangi on SFN-induced dephosphorylation of p38MAPK were analyzed. We found that the combination of SFN and Sangi decreased p38MAPK phosphorylation in BC3 cells compared with SFN alone and Sangi alone (Fig. 2L). In contrast, we found no alterations in p38MAPK phosphorylation in DG75 cells subjected to any of the treatments. Interestingly, MG132 alone and the combination of SFN with MG132 increased p38MAPK phosphorylation in both DG75 and BC3 cells compared to untreated cells and cells treated with SFN alone (Fig. 2F). These results indicated that MG132 induced p38MAPK phosphorylation in both PEL and KSHV-uninfected B-cells. We also evaluated the AKT phosphorylation status after treatment of DG75 and BC3 cells with a combination of SFN and MG132 or Sangi, however, remarkable changes were not observed (data not shown).

NF\(\kappa\)B, AKT, p38MAPK and ERK signaling are known...
to be constitutively and/or transiently activated in PEL cells in order to maintain the malignant phenotype and to ensure PEL cell survival.\(^6\)\(^-\)\(^10\) In particular, KSHV activates AKT and p38MAPK signaling, allowing the establishment of a KSHV infection, cell growth and survival of PEL. In this study, we demonstrated SFN represses p38MAPK and AKT signaling by inhibiting p38MAPK and AKT phosphorylation, which results in PEL cell apoptosis (Fig. 1). We previously found that MG132 suppressed NF-κB signaling and induced apoptosis in PEL cells by inhibiting proteasome-dependent degradation of IκBα.\(^6\)\(^-\)\(^8\) We also reported that Sangi induced apoptosis in PEL cells by suppressing ERK and AKT signaling.\(^8\) The suppression of p38MAPK signaling of PEL cells by MG132 and Sangi were not observed in previous studies.\(^6\)\(^-\)\(^8\) Our results demonstrated that Sangi and SFN acted in a synergistic manner to exert cytotoxic effects induced by the combination of SFN with Sangi.

SFN interferes with numerous cancer-related signaling pathways, resulting in an apoptosis induction.\(^11\)\(^,\)\(^12\) SFN has been reported to inhibit AKT,\(^13\) ERK,\(^14\) p38MAPK signaling.\(^15\) The thiol group of Cys in some cellular proteins is thought to bind to the isothiocyanate (–N=C=S) of SFN by the nucleophilic reaction. In fact, the isothiocyanate of SFN is covalently conjugated to the Cys of glutathione and Kelch-like ECH-associated protein 1 (Keap1), resulting in activation of antioxidant signaling.\(^11\)\(^,\)\(^12\) Thus, we could speculate that SFN might inhibit p38MAPK and AKT signaling by covalent binding between SNF and p38MAPK, AKT or their upstream molecules.

We found that drug combinations can disrupt multiple signaling pathways in PEL cells, which improves their survival. The combined therapy of SFN with other drugs, which have different targets, might be effective treatments for PEL. Together, our data show that SFN induces apoptosis in PEL cells by repression of p38MAPK and AKT phosphorylation. Moreover, the addition of MG132 or Sangi enhanced the cytotoxic activity of SFN in a synergetic manner. These results revealed that SFN has clinical utility as a novel treatment for PEL.

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Conflict of Interest The authors declare no conflict of interest.

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