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Effects of sodium stibogluconate on differentiation and proliferation of human myeloid leukemia cell lines *in vitro*

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PTPases are key signaling molecules and targets for developing novel therapeutics. We have studied the *in vitro* biological activity of PTPase inhibitor sodium stibogluconate (SS) on differentiation and proliferation of myeloid leukemia cell lines (NB4, HL-60 and U937). SS (250 μg/ml, 6 days) induced 87% of NB4 cells to reduce nitroblue tetrazolium (NBT), in comparison to the 90% induced by ATRA (1 μM, 6 days). SS treatment of NB4 cells resulted in an increase of CD11b expression and of a morphologically more mature population, coincident with growth arrest at S phase and increased cell death. The effect of SS on NB4 differentiation was irreversible and required continuous drug exposure. SS (400 μg/ml, 6 days) induced 60% and 55% of NBT-positive cells in HL-60 and U937 cell lines, which were augmented in the presence of GM-CSF (25 ng/ml) to levels (85% and 81%, respectively) comparable to those induced by ATRA. SS induced increased tyrosine phosphorylation of cellular proteins in the AML cell lines and inactivated SHP-1 PTPase in NB4 cells, consistent with SS functioning as a PTPase inhibitor in the leukemia cells. These results provide the first evidence of an anti-leukemia activity of SS as a PTPase inhibitor.
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Introduction

Acute myeloid leukemia (AML) is characterized by the accumulation of myeloid blast cells that are arrested at various differentiation stages and unable to terminally differentiate.[1] Based on morphology, cytochemistry, immunological markers and cytogenetics, AML can be divided into distinct subclasses.[2–5] Treatment for most subclasses of AML is unsatisfactory.[1] It usually includes intensive chemotherapy administered as induction treatment to induce complete hematological remission and consolidation therapy to eradicate residual disease. Consolidation therapy with chemotherapy alone or in combination with autologous stem cell transplantation is associated with a relatively high risk of relapse and a long-term disease-free survival of less than 50%. Consolidation therapy with allotransplantation has a lower relapse risk but a higher treatment-related mortality.[1]

Potential of differentiation induction therapy in AML treatment is highlighted by the recent success of all-*trans* retinoic acid (ATRA) in the treatment of acute promyelocytic leukemia (APL, M3 subclass).[6] ATRA has been shown to induce complete remission and to increase long-term APL-free survival exceeding 75%.[7] This therapeutic effect of ATRA derives from its activity in inducing terminal differentiation of APL cells through its binding to aberrantly generated chimeric proteins of retinoic acid receptor a (RARα) that results in degradation of the chimeric proteins and altered transcription regulation.[6] As generation of chimeric proteins of RARα is restricted to APL cells,[1] differentiation induction therapy with ATRA showed only limited benefit in the treatment of other AML subclasses[1]. Moreover, ATRA differentiation induction therapy works well in APL cases with t(15;17) translocation but showed little or no effect on those with t(11;17) or t(5;17) translocation.[6] Therapeutic use of ATRA is further compromised by serious systemic toxicity[8] and induced ATRA resistance.[9] Nevertheless, the marked success of ATRA in the subgroup of APL cases has provided evidence indicating the efficacy of differentiation induction therapy in AML treatment and prompted extensive efforts to identify other differentiation induction therapeutics. Several candidates were recently reported, including histone deacetylase inhibitors.[10] Although a number of hematopoietic growth factors and cytokines used alone or in combination with other reagents are known to promote myeloid differentiation,[11] their clinical usage in AML treatment is controversial due to marked variations in the responses of AML cells to ligands.[12–15] Arsenic trioxide and its derivatives have also been shown to have anti-leukemia activity but function via a complex mechanism involving induction of apoptosis.[16]

Several lines of evidence have indicated that AML cell differentiation is affected by cellular protein tyrosine phosphorylation regulated by the balance of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases). Granulocytic maturation of HL-60 leukemia cells (M2 subclass) was shown to produce a decrease in cellular protein tyrosine phosphorylation and increases in both tyrosine kinase and protein phosphotyrosine phosphatase activities.[17] Hematopoietic protein tyrosine phosphatase (HePTP) amplification and overexpression were found in AML cells and cell lines and may contribute to abnormal AML cell growth and arrest of differentiation.[18] The involvement of hematopoietic cell phosphatase SHP-1 was indicated by its increased expression during HL-60 cell differentiation[19] and its inhibition of Epo-induced differentiation of J2E leukemic cells.[20] Interestingly, PTK inhibitor STI571 was shown to enhance ATRA-induced differentiation of APL cells although alone it had no differentiation induction activity.[21] So far, induction of AML cell differentiation by PTPase inhibitors has not been reported.

Sodium stibogluconate (SS) has been used for decades in the treatment of leishmaniasis with its mechanism of action poorly understood.[22] It was known that SS selectively kills intracellular leishmania but not the free living form of the protozoa[23] and that its anti-leishmania activity was severely impaired in immune deficient hosts.[24] These observations suggest that SS targets host cellular molecules that mediate its activity. Our recent studies demonstrate for the first time that SS is a potent inhibitor of PTPases and augments signaling of various hematopoietic growth factors and cytokines.[25] This mode of action of SS provides a rational explanation for its selective activity against intracellular parasites and its depen-

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dency on host immunity. It also suggests novel applications of drug as a clinically usable PTPase inhibitor. To explore the potential of SS in differentiation induction therapy in the treatment of AML, we have determined its effects on differentiation and proliferation of human AML cell lines *in vitro*.

Materials and methods

Reagents

All-*trans* retinoic acid (ATRA), nitroblue tetrazolium (NBT), and 12-O-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma (St Louis, MO, USA). Sodium stibogluconate (SS) (25) and recombinant human GM-CSF have been described previously.[26] Rabbit anti-SHP-1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and phosphotyrosine peptide substrate (UBI, Lake Placid, NY, USA) were purchased from commercial sources.

Cell lines, cell culture and cell proliferation assay

The NB4 cell line[27] was a gift from Dr Dan Lindner of the Cleveland Clinic Foundation (CCF). HL-60[28] and U937[29] cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). These human AML cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). For cell proliferation assays, cells were cultured at 37°C in 10% FCS medium containing various amounts of SS for 6 days. The cell numbers in the cultures were determined by an MTT assay.[30]

Studies of induction of differentiation

Differentiation of AML cell lines was assessed by their ability to produce superoxide as measured by reduction of NBT to formazan and by analysis of expression of CD11b surface marker by flow cytometry. For NBT reduction,[31] each cell suspension was mixed with an equal volume of solution containing 1 mg/ml of NBT (Sigma) and 2.5 μg/ml of TPA for 30 min at 37°C. After incubation, cells containing the purple formazan deposits and cells devoid of NBT-reducing activity (white cells) in each sample were determined by counting 200 cells under microscope. Data were expressed as percentage of NBT-positive (NBT^+) cells based on the following ratio: purple cells/purple + white cells. For analysis of cell surface antigens, cells were exposed to phycoerythrin (PE)-conjugated murine anti-human CD11b (DAKO Corp, Carpinteria, CA, USA). Analysis of fluorescence was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). For morphologic evaluation, cells on slides were fixed in methanol for 10 min, incubated in Giemsa staining solution for 20 min, washed in water and examined under immersion microscopy (×60). Two hundred cells/slide were examined to derive the percentage of cells with morphologic differentiation.

Cell cycle analysis

The cell cycle was analyzed by flow cytometry after 3 days of culture of NB4 cells in the absence or presence of SS (250 μg/ml) or ATRA (1 μM). Briefly, the cells were fixed in cold ethanol and incubated for 30 min at 4°C in the dark with a solution of 50 mg/ml propidium iodide, 1 mg/ml RNase and 0.1% NP-40. Analysis was performed immediately after staining using the CELLFIT program (Becton Dickinson).

Detection of apoptotic cells by Annexin V/propidium iodide assay

Annexin V staining of exposed membrane phospholipid phosphatidylserine (PS) was done using the Annexin V assay kit (Pharmingen, San Diego, CA, USA). Briefly, NB4 cells were cultured in 10% FCS RPMI 1640 medium in the absence or presence of SS (250 μg/ml) or ATRA (1 μM) for 3 days. Cells were then washed in PBS twice and stained in binding buffer (10 mM Hepes, pH 7.4; 140 mM NaCl; 2.5 mM CaCl2) containing Annexin V-FITC and propidium iodide for 15 min. The reaction was stopped by adding 10 volumes of binding buffer. Apoptosis within the cell population was analyzed without gating by FacsVantage (Becton Dickinson) with a total of 10 000 events acquired for each sample. Histograms were generated for each sample based on identical parameters.

Induction of cellular protein phosphorylation and immunocomplex PTPase assays

For induction of cellular protein phosphorylation by SS, cells were incubated in 0.1% FCS RPMI 1640 medium at 37°C for 16 h. The cells were then washed twice in RPMI 1640 medium and incubated with sodium stibogluconate for 5 min prior to termination by lysing cells in cold lysis buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 0.2 mM Na_3VO_4; 20 mm NaF; 1% NP40; 2 mM PMSF; 20 mg/ml of Aprotinin and 1 mM of sodium molybdic acid). Total cell lysates were separated in SDS-PAGE gels, blotted on to nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA), probed with specific antibodies and detected using an enhanced chemiluminescence kit (ECL, Amersham, Arlington Heights, IL, USA).[25]

Immunocomplex PTPase assays were performed to assess the effects of SS on intracellular SHP-1 PTPase in NB4 cells. SHP-1 protein was immunoprecipitated from NB4 cells that were untreated or treated with various amounts of SS for 5 min and then lysed in cold lysis buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 1% NP40; 2 mM PMSF; 20 μg/ml of Aprotinin). Each sample of the immunocomplexes was split into two portions, one of which containing 10% of the materials that was used for immunoblotting to assess the amounts of SHP-1 in the immunocomplexes. The remaining portion was further divided equally into three parts and incubated in 50 μl of Tris buffer containing 0.2 mM of phosphotyrosine peptide substrate (UBI) at 25°C for 16 h. Malachite green solution (100 μl) was then added to each reaction which was then incubated at 22°C for 5 min prior to measurement of OD660 to quantify the amounts of free phosphate cleaved by the PTPases from the peptide substrate.[25] Relative PTPase activity was calculated (OD660 of SHP-1 from SS-treated NB4 cells/OD660 of SHP-1 from untreated NB4 cells × %).

Results

SS induces differentiation of AML cell line NB4 in a dose- and time-dependent manner

NB4 is a human AML cell line derived from an APL patient and can be induced to differentiate into granulocytes by ATRA.[27] To explore the potential of SS in differentiation induction therapy for AML, we initially determined the activity of the drug in inducing differentiation of NB4 cells into more mature granulocyte-like cells by NBT reduction assays, CD11b antigen expression and morphologic evaluation.

SS induced an increase of NBT-positive NB4 cells in a dose- and time-dependent manner (Figure 1). SS showed such an activity at all of the dosages (10 to 400 μg/ml) that were tested in day 3 or day 6 culture (Figure 1a). The optimal dosage was at 250 μg/ml which induced 87% of NB4 cells cultured in the presence of SS for 6 days (Figure 1a). At this dosage, SS-induced NBT-positive cells were detectable after cells were treated with the drug for the first 24 h, increased further during the following days and reached 87% by day 6 (Figure 1b). NB4 cells treated with ATRA (1 μM) for 6 days also reached similar levels of NBT-positive cells under comparable conditions with NBT staining in individual cells generally stronger than that in SS-induced cells (Figure 1b). SS-treated NB4 cells also showed an increase of CD11b expression (Figure 1c) and of morphologically more mature cells that were characterized by polylobular nuclei, decreased nuclear:cytoplasmic ratio and decreased cytoplasm staining (Figure 1d).

SS-induced NB4 cell differentiation associates with cell growth arrest at S phase and increased cell death

We next determined the effect of SS on NB-4 cell growth by MTT assays. Proliferation of NB4 cells was markedly inhibited in the presence of SS at all the dosages that were examined (12.5–400 μg/ml) (Figure 2a). Cell DNA content analysis (Figure 2) showed a significant increase of cells at S phase in the NB4 cells treated with SS (250 μg/ml) for 3 days (Figure 2b). In contrast, NB4 cells cultured in the presence of ATRA (1 μM) for 3 days were arrested at G1 phase (Figure 2b), consistent with a previous report.[32] A substantial population of NB4 cells cultured in the presence of SS (250 μg/ml) for 6 days was stained positive by Annexin V, suggesting that the cells were dying through apoptosis (Figure 2c). These results demonstrated that SS induced NB4 cell growth arrest at S phase and had a cytotoxic effect against the cells.

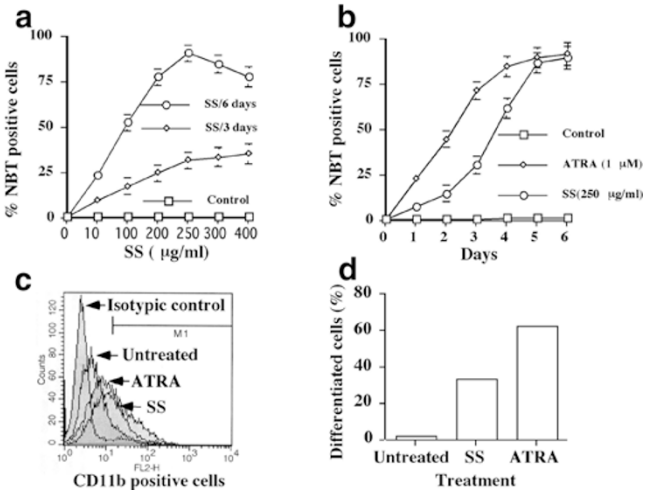

Figure 1 SS induces NB4 differentiation in a dose- and time-dependent manner. (a) NB4 cells were cultured in the absence or presence of various amounts of SS for 3 or 6 days. The percentage of NBT-positive cells in NB4 cells cultures was determined. The data represent the mean ± s.d. values of triplicate samples. (b) Percentage of NBT-positive cells in NB4 cells cultured in the presence of 1 μM of ATRA or 250 μg/ml of SS for various time points. Data represent the mean ± s.d. of triplicate samples. (c) CD11b-positive cells in NB4 cells cultured in the presence of 250 μg/ml of SS or ATRA (1 mM) for 3 days as determined by FACS analysis. (d) Percentage of morphologically mature cells in NB4 cells untreated or treated with SS (250 μg/ml) or ATRA (1 μM) for 3 days.

SS-induced NB4 differentiation is irreversible and requires continuous exposure to the drug for optimal induction

We next investigated whether SS-induced NB4 differentiation would be reversed in the absence of the drug. NB4 cells cultured in the presence of SS (10 μg/ml or 100 μg/ml) for 6 days were washed and re-suspended in medium without the drug. The cells were then cultured for 6 days with the numbers of NBT-positive cells determined daily. As shown in Figure 3a, the percentage of NBT-positive cells remained largely consistent during the 6 day period, demonstrating that SS-induced NB4 differentiation was not reversed in the absence of the drug. Under comparable conditions, ATRA-induced NB4 differentiation showed a similar characteristic as previously reported.[27]

To determine whether induction of NB4 cell differentiation requires long-term exposure to the drug, NB4 cells were cultured in the presence of the drug (100 μg/ml) for 0.5 to 24 h, then washed and cultured in medium without the drug for 6 days prior to NBT staining. A linear increase of NBT-positive cells was detected in NB4 cells exposed to the drug for 0.5 to 24 h with maximal increase (16%) at 24 h (Figure 3b). Thus NB4 cell differentiation was inducible following short exposure to the drug. However, the 16% NBT-positive cells induced by exposing to the drug for 24 h was substantially less than the 52% level in NB4 cells cultured in the presence of SS (100 μg/ml) for 6 days (Figure 1a). Since the percentage of differentiated cells in the culture was directly related to the length of exposure time to SS (Figure 1b), the results together indicated that optimal induction of NB4 cell differentiation by SS requires continuous drug exposure. Similarly, NB4 cell differentiation induced by short exposure to the ATRA (Figure 3b) was modest in comparison to that of long-term exposure (Figure 1b).

SS induces differentiation of HL-60 and U937 cell lines

To investigate whether the differentiation induction activity of SS was unique to NB4 cells, we determined the effect of the drug in AML cell lines HL-60 and U937. HL-60 and U937 cells were cultured in the absence or presence of various amounts of SS for different times. The percentage of NBT-positive cells in the culture was determined as an indicator of cell differentiation.

SS induced differentiation of HL-60 and U937 cells in a dose- and time-dependent manner (Figure 4). The optimal dosage of SS in inducing differentiation of HL-60 and U937 cells was 400 μg/ml under the experimental conditions in day

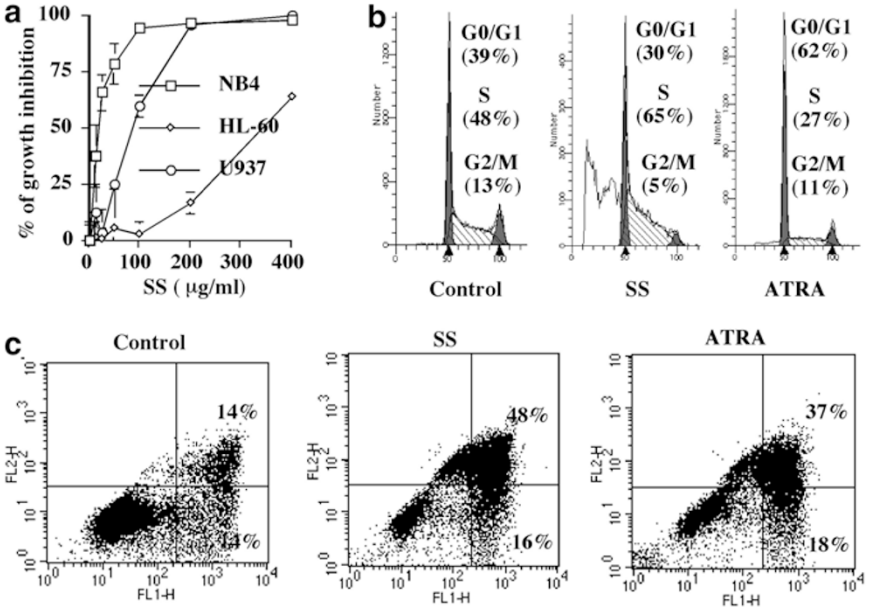

Figure 2 SS-induced NB4 cell differentiation associates with growth arrest at S phase and cell death. (a) NB4, HL-60 and U937 cells were cultured in the absence or presence of various amounts of SS for 6 days. Cell growth was determined by MTT assays. Percentage of cell growth inhibition was calculated. Data represent the mean ± s.d.of triplicate samples. (b) NB4 cells cultured for 3 days in the absence or presence of SS (250 μg/ml) or ATRA (1 μM) were stained with propidium iodide and analyzed for cellular DNA content to calculate the percentage of cells at G0/G1, S or G2/M phases. (c) NB4 cells cultured for 3 days in the absence or presence of SS (250 μg/ml) or ATRA (1 μM) were stained with propidium iodide (PI, y-axis) and Annexin V FITC (x-axis). Flow cytometric plots show binding of Annexin V, indicating exposure of phosphatidylserine residues on the cell membrane (early stages of apoptosis), and PI labeling, indicating membrane permeabilization (late-stage cell death).

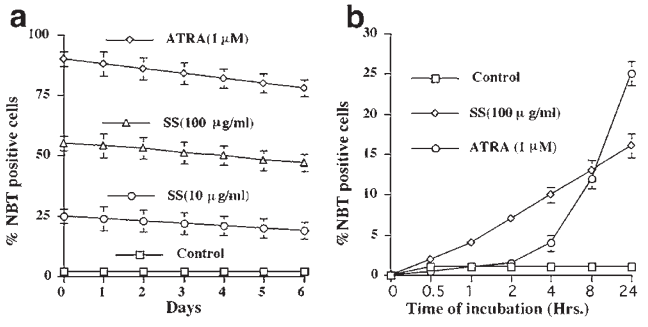

Figure 3 SS-induced NB4 differentiation is irreversible and could be triggered by short exposure to the drug. (a) NB4 cells cultured in the absence or presence of SS (100 μg/ml) or ATRA (1 μM) for 6 days were washed, resuspended in medium without the drug and cultured for 6 days with the percentage of NBT-positive cells determined daily. (b) NB4 cells were incubated with SS (100 μg/ml) or ATRA (1 μM) for 0.5 to 24 h. The cells were then washed, resuspended in medium without the drugs and cultured for 6 days. The percentage of NBT-positive cells in the day 6 cultures were determined. Data represent the mean ± s.d. of triplicate samples.

6 culture (Figure 4a and c). At this dosage, the SS-induced differentiation (approximately 60%) of HL-60 and U937 cells was less than that induced by ATRA (90% for HL60 and 72% for U937) in day 6 culture (Figure 4b and d). Similar to NB4 cells, the percentage of differentiated cells of HL-60 and U937 increased proportionally with prolonged culture in the presence of SS (Figure 4b and d), indicating a requirement of continuous drug exposure for optimal differentiation induction. The PTPase inhibitor also showed a growth inhibition activity against the two AML cell lines. At the optimal dosage (400 μg/ml) of the drug for differentiation induction in the two cell

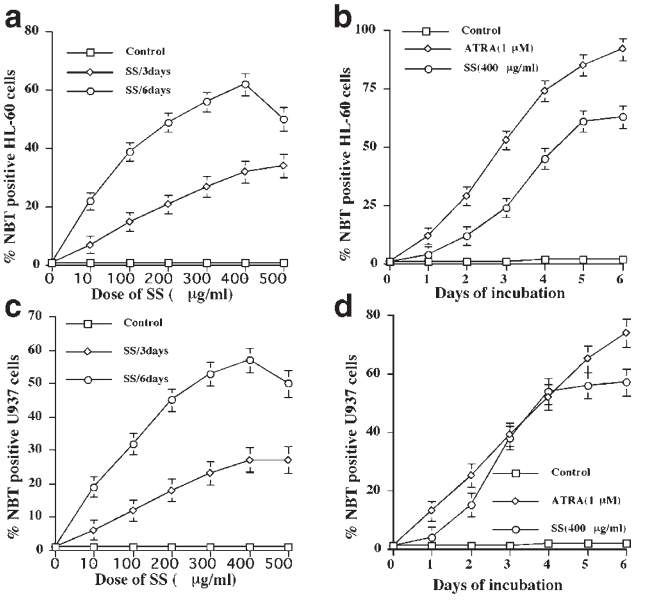

Figure 4 SS induces differentiation of HL-60 and U937 cells.(a). The percentage of NBT-positive cells in HL-60 cells cultured in the absence or presence of various amounts of SS for 3 or 6 days. (b) The percentage of NBT-positive cells in HL-60 cultured in the presence of ATRA (1 μM) or SS (400 μg/ml) for 0–6 days. (c) Percentage of NBT-positive cells in U937 cells cultured in the absence or presence of various amounts of SS for 6 days. (d). The percentage of NBT-positive cells in U937 cultured in the presence of ATRA (1 μM) or SS (400 μg/ml) for 0–6 days. The data represent the mean ± s.d. of triplicate samples.

lines, SS achieved 97% growth inhibition of U937 cells and 63% inhibition of HL-60 cells in day 6 cultures (Figure 2a).

SS-induced differentiation of HL-60 and U937 is augmented by GM-CSF

Our recent studies showed that SS augments signaling initiated by GM-CSF,[25] which is known to promote myeloid cell proliferation and differentiation.[31] We therefore determined the effect of the drug in combination with the cytokine in inducing differentiation of HL-60 and U937 cells. HL-60 and U937 cells were cultured in the presence of SS (400 μg/ml), GM-CSF (25 ng/ml) or both for 1–6 days with the percentage of NBT-positive cells determined daily.

SS-induced differentiation of HL-60 and U937 was augmented by GM-CSF to levels nearly equal or higher than those induced by ATRA (Figure 5). Consistent with previous reports,[33] GM-CSF alone showed a minor effect on HL-60 (Figure 5a) and U937 (Figure 5b) differentiation, with maximal increase of NBT-positive cells (8–10%) at day 6. Interestingly, the percentage of NBT-positive cells in HL-60 cultured in the presence of both GM-CSF and SS was increased to 83% compared to 60% with SS alone (Figure 5a) or 90% with ATRA alone (Figure 4b). More dramatically, the combination of GM-CSF and SS in U937 cells induced 80% cell differentiation, which was higher than that of SS alone (55%) (Figure 5b) or ATRA alone (73%) (Figure 4d). In contrast, GM-CSF alone showed no detectable effect on NB-4 cell differentiation (data not shown), consistent with a previous report,[34] and failed to augment SS-induced NB4 cell differentiation under comparable conditions (data not shown).

SS induces tyrosine phosphorylation of cellular proteins and inactavates SHP-1 in AML cells

To assess whether SS functions as a PTPase inhibitor in AML cell lines, we determined cellular protein tyrosine phosphorylation in NB4, U937 and HL-60 cells untreated or treated with various amounts of SS.

Tyrosine phosphorylation of two cellular proteins approximately 30 and 32 kDa in NB4 cells was modestly increased following SS treatment in a dose-dependent manner (Figure 6a, lanes 2–4). Increased tyrosine phosphorylation of proteins of similar sizes was also evident in SS-treated U937 and HL-60 cells (Figure 6a, lanes 6–8 and 10–12). The identities of the two proteins have not been determined. SS treatment had no obvious effect on other major phosphotyrosine proteins in the cells as assessed via shorter exposures of Figure 6a (data not shown). These results were consistent with SS inhibition of selective PTPases in the AML cell lines.

The effect of SS on SHP-1 PTPase in NB4 cells was further investigated as a more direct indicator of inhibition of intracellular PTPases by the drug. SHP-1 immunoprecipitated from NB4 cells treated with SS for 5 min showed little activity in comparison to that from untreated NB4 cells (Figure 6b). These results demonstrated that SHP-1 in NB4 cells was inactivated following the brief SS treatment, consistent with the sensitive nature of recombinant SHP-1 to the inhibitor *in vitro*.[25]

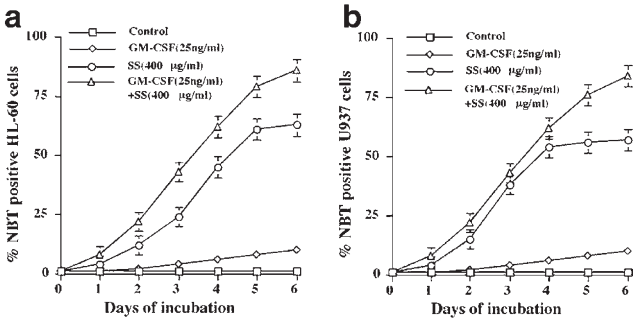

Figure 5 GM-CSF augments SS-induced differentiation of HL-60 and U937 cells. HL-60 (a) or U937 (b) cells were cultured in the absence or presence of GM-CSF, SS or both for various time points with the percentage of NBT-positive cells determined daily. Data represent the mean ± s.d. of triplicate samples.

Discussion

Herein we provide the first evidence indicating the potential of PTPase inhibitor SS in differentiation induction therapy in AML treatment. We demonstrate that SS, a drug used for leishmaniasis and a PTPase inhibitor, induces differentiation of AML cell lines NB4, HL-60 and U937 *in vitro*. Our data showed that SS induces granulocyte-like maturation of NB4, HL-60 and U937 cells as indicated by the increase of NBT-positive cells and by the increase of CD11b-positive cells and morphologically more mature cells (NB4). This activity of the drug was detectable at low dosage of the drug following relatively short exposure. We further demonstrate that SS-induced differentiation is irreversible and associates with growth arrest and cell death via, probably, apoptosis. These results demonstrate an anti-leukemia activity of the drug in AML cell lines *in vitro* and suggest SS as a candidate therapeutic for AML treatment.

Our results suggest that SS might be effective in inducing differentiation of AML cells of different subclasses. This is indicated by its differentiation induction activity in the AML cell lines that represent M2 (HL-60), M3 (NB4) and M5 (U937) subclasses.[35] Moreover, we provide evidence that the anti-leukemia activity of SS is likely mediated via a mechanism involving inhibiting PTPases and different from that of ATRA. SS functioned as a PTPase inhibitor in the AML cell lines as indicated by its inactivation of intracellular SHP-1 in NB4 cells and by its induction of increased tyrosine phosphorylation in certain cellular proteins in AML cells (Figure 6). In addition, SS had no detectable effect on the expression levels of PML/RARα chimeric protein in NB4 cells and did not synergize with ATRA in differentiation induction (our unpublished data) whereas ATRA is known to target the PML/RARα chimeric protein and induces its degradation in NB4 cells.[36] Such a distinct mechanism of SS against leukemic cells suggests that SS may be beneficial in AML and in cases of APL unresponsive or developing resistance to ATRA treatment. Further studies are obviously needed to evaluate its effects against primary leukemia cells.

Results from our studies provide insights into the identity of SS-targeted PTPase in mediating SS effects against AML cells. Among the 100 or so PTPases encoded in mammalian genomes,[37] a significant number are expressed in myeloid cells[38] although their roles in myeloid differentiation and myeloid cell proliferation have not been fully characterized. SHP-1 is apparently an SS-targeted PTPase in AML cells (Figure 6).

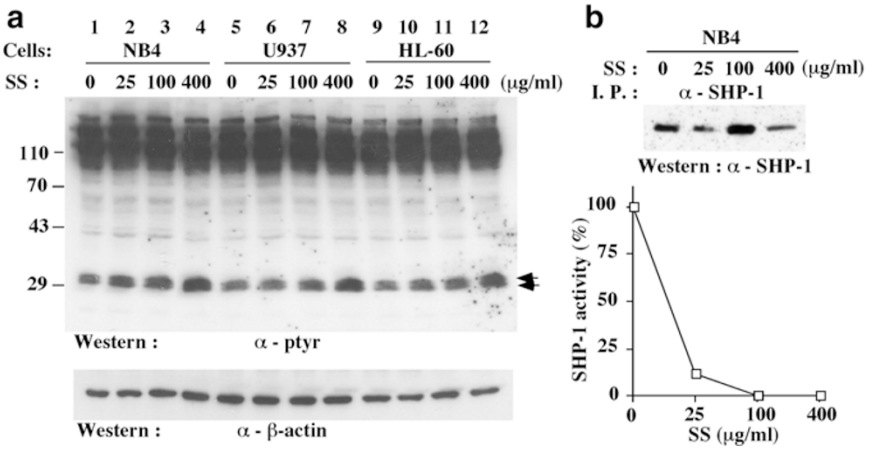

Figure 6 SS induces tyrosine phosphorylation of cellular proteins in AML cell lines and inactivates intracellular SHP-1 in NB4 cells. (a) NB4, U937 and HL-60 cells were cultured in the absence or presence of various amounts of SS. Total cell lysates of the cells were analyzed by SDS-PAGE/Western blotting with an antibody against phosphotyrosine (α-ptyr) or an anti-β-actin antibody as indicated. Positions of protein size markers (kDa) are indicated on the left. Two proteins with increased tyrosine phosphorylation following SS treatment are indicated by the arrows. (b) Relative PTPase activity of SHP-1 immunoprecipitated from NB4 cells untreated or treated with SS (lower panel). Data represent mean ± s.d. values of triplicate samples. The amounts of SHP-1 protein in the immunocomplexes were quantified by immunoblotting (higher panel).

Given its known activity in down-regulating Jak/Stat signaling initiated by GM-CSF[39] that promotes myeloid differentiation,[11] its inactivation could be involved in augmentation of SS-induced differentiation of AML cells. However, it is unlikely to be the key PTPase mediating SS-induced differentiation of AML cells because inactivation of SHP-1 in mouse models does not associate with accelerated myeloid differentiation.[40] We propose that the key SS target in AML differentiation is among aberrantly activated PTPases in AML cells that promote proliferation and block differentiation. In this regard, the involvement of amplification and over-expression of HePTP in AML[18] is interesting and suggests it as a candidate target of the drug. Characterization of PTPase expression profiles of SS-sensitive and SS-resistant AML cell lines and determination of the sensitivities of the PTPases to SS might help to define the putative SS target PTPase in AML differentiation. Similarly, identification of the SS-induced phosphotyrosine proteins in AML cells in future studies might also provide insights into the mechanism of action of SS in inducing AML cell differentiation.

The optimal dosage of SS for inducing differentiation of NB4 and HL-60/U937 cells is 250 μg/ml and 400 μg/ml, respectively. The standard dosage for leishmania treatment is 10–20 mg/kg/day resulting in 10 μg/ml or more serum levels.[22] However, higher drug dosages may be clinically achievable and tolerated since doses as high as 80–143 mg/kg have been used in leishmania treatment.[41] Nevertheless, even standard dosage of SS may have certain therapeutic benefit as the drug at lower dosages (eg 10 μg/ml) showed differentiation induction activity in AML cells (Figures 1 and 4). Further studies using mouse models of AML are needed to verify the differentiation induction activity of the drug and to determine the toxicity of the drug at the optimal dosages *in vivo*.

The observation that GM-CSF augments SS-induced differentiation of HL-60 and U937 suggests the potential clinical use of the two reagents in combination in AML treatment (Figure 5). Such an interaction between SS and GM-CSF is not unexpected given the activity of the drug in augmenting GM-CSF signaling[25] and the biological effect of the cytokine on myeloid cells.[11] However, combination usage of SS and GM-CSF may only benefit a subgroup of AML cases as a positive interaction between the two reagents in differentiation induction was not detected in NB4 cells, which were not responsive to the cytokine.[34] Moreover, SS may also interact with other cytokines in differentiation induction of AML cells. G-CSF and IFNs were reported to potentiate differentiation of AML cells.[42,43] Like GM-CSF, the two cytokines signal through the Jak/Stat pathway[44] that could be augmented by SS.[25] The existence of such potential interactions is being examined in our ongoing studies.

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