Activation of C-Kit by Stem Cell Factor Induces Radioresistance to Apoptosis through ERK-dependent Expression of Survivin in HL60 Cells

Seyed JALAL HOSSEINIMEHR1**, Osamu INANAMI1, Taku HAMASU1, Momoko TAKAHASHI1, Ikuo KASHIWAKURA2, Taketoshi ASANUMA1 and Mikinori KUWABARA1*

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We investigated the effect of SCF, a c-kit ligand, on the radiosensitivity of HL60 cells. X-ray-induced apoptosis in HL60 cells was significantly lower in the presence of SCF than in the absence of SCF. This attenuation of X-ray-induced apoptosis by SCF was abolished by PD98059 (an ERK inhibitor), but not by wortmannin (a PI3-K inhibitor) or GF109203X (a PKC inhibitor). The expression of phospho-ERK1/2 (active form) and the ERK1/2-regulated expression of survivin were found to increase in cells treated with X irradiation and SCF. However, X irradiation alone induced down-regulation of the expression of phospho-ERK1/2. Our findings suggest that activation of c-kit by SCF confers radioresistance through up-regulation of ERK-dependent survivin expression in HL60 cells.

INTRODUCTION

The proto-oncogene c-kit encodes a transmembrane tyrosine kinase receptor (c-kit/CD117) related to the platelet-derived growth factor (PDGF)/colony-stimulating factor 1 (CSF-1) receptor subfamily. The c-kit is thought to play an important role in hematopoiesis, spermatogenesis, melanogenesis and carcinogenesis. More recently, it was shown that expression of an activating mutation of c-kit in acute myeloid leukemia (AML) cells, mastocytosis and germ cell tumors acquired a growth advantage and resistance to radiation and chemotherapy. Kashiwakura et al. found that stem cell factor (SCF), a c-kit ligand, enhanced the thrombopoietin (TPO)-induced cell growth in CD34+ CFU-megakaryocytes and that combination treatment with SCF and TPO synergistically protected X-irradiated CD34+ CFU-megakaryocytes against X-ray-induced death. Combinated treatment with CSF and TPO led to the activation of extracellular signal-regulated protein kinase (ERK) and the suppression of caspase 3 in X-irradiated CD34+ cells. When PD98059, a specific inhibitor of ERK, was used, the combination had less effect on the clonal growth of X-irradiated CD34+ CFU-megakaryocytes. However, the addition of wortmannin, a specific inhibitor of the phosphatidylinositol-3 kinase (PI3-K) pathway, did not alter the synergistic action of TPO plus SCF. Recently, in 16 of 18 primary AML and 8 leukemic cell lines, the expression of survivin, which is a characterized gene and a member of the inhibitor of apoptosis protein (IAP) family, was reported to inhibit apoptosis through a pathway different from that involving the BCL-2 family. Furthermore, the incubation of OCI-AML cells or HL60 cells with SCF enhanced the ERK-dependent expression of survivin. However, the role of survivin in c-kit-mediated radioresistance and chemoresistance is still unclear.

In the present study, to fully understand how c-kit-activated leukemia cells become radioresistant, we have investigated effects of SCF on radiation-induced apoptosis in HL60 cells and examined the relationship between radiation-induced apoptosis and SCF-induced ERK-dependent expression of survivin.
MATERIALS AND METHODS

Materials
Granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF) were purchased from Calbiochem (San Diego, CA). Propidium iodide (PI) was purchased from Sigma (St Louis, Mo), RPMI 1640 was from Gibco/BRL, Life Technologies (Rockville, MD). Nitrocellulose membranes were from ADVANTEC Toyo (Tokyo, Japan). Acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) was from the Peptide Institute Inc. (Osaka, Japan). PD98059 (ERK inhibitor), wortmannin (PI3-K inhibitor) and GF109203X (PKC inhibitor) were from Calbiochem (San Diego, CA). Anti-p44/42 MAP kinase (ERK1/2) and anti-phosphorylated p44/42 (phospho-ERK1/2) MAP kinase antibodies were from Cell Signaling Technology (Beverly, MA). Anti-survivin and anti-BCL-2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture
Human leukemia cell line HL60 was purchased from RIKEN Cell Bank (Tsukuba, Japan) and was grown in RPMI 1640 medium containing 10% fetal calf serum at 37°C in 5% CO2.

Treatments of cells with drugs and X irradiation
Cells were treated with growth medium containing 100 ng/ml of SCF without or with 20 µM Ac-DEVD-CHO, 25 µM PD98059 and 200 nM wortmannin and 1 µM GF109203X at 37°C for 1 h before X irradiation. X irradiation of cells was performed with an X ray generator (2.0 mm Al filter, 200 kVp, 20 mA, Shimadzu HF-320, Kyoto, Japan) at a dose rate of 3.0 Gy/min at room temperature, which was determined by Fricke’s chemical dosimeter.

Fluorescence microscopic observation of apoptotic cells
Apoptotic cells were accessed as previously described. Briefly, cells incubated for the indicated periods of time after X irradiation were collected by centrifugation at 1000 rpm for 5 min at 4°C. The pellet was washed with PBS [-] two times and resuspended in a 15 µl of Laemmli’s sample buffer (62.0 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.003% bromophenol blue). Proteins in the solution were separated by SDS-PAGE after boiling for 3 min and proteins in gel were then transferred to a nitrocellulose membrane. The membrane was reacted with an anti-ERK1/2, anti-phosphorylated ERK1/2, anti-BCL-2 or anti-survivin antibody in TBST buffer (10 mM Tris-HCl [pH 7.4], 0.1 M NaCl, 0.1% Tween-20) containing 5% low fat Skim milk overnight at 4°C. The ERK1/2, phosphorylated ERK1/2, BCL-2 and survivin antibodies were detected by a method using HRP-conjugated anti-rabbit or mouse IgG antibodies with a chemiluminescence detection kit (PerkinElmer Life Sciences Inc., Boston, MA).

RESULTS AND DISCUSSION

Figure 1A shows the effects of GM-CSF, G-CSF and SCF on X-ray-induced apoptosis in HL60 cells. When HL60 cells were exposed to 5 Gy of X rays, the typical morphological alterations characteristic of apoptosis, including chromatin condensation and nuclear fragmentation, gradually increased after X irradiation. Incubation with 100 ng/ml of SCF, but not that with GM-CSF and G-CSF, significantly attenuated X-ray-induced apoptosis 48 and 72 h after X irradiation. As shown in Fig. 1B, this inhibition of X-ray-induced apoptosis by SCF occurred in a concentration-dependent manner. To study signal transduction pathways responsible for the attenuation of X-ray-induced apoptosis by SCF, we next examined the effects of various pharmacological inhibitors on X-ray-induced apoptosis. Figure 1C shows the effects of caspase-3, ERK1/2, PI3-K and PKC inhibitors on X-ray-induced apoptosis in HL60 cells with SCF. Induction of X-ray-induced apoptosis was considerably inhibited by Ac-DEVD-CHO, indicating the occurrence of caspase 3-dependent apoptosis in HL60 cells. The amount of X-ray-induced apoptosis with SCF and PD98059 was similar to that of X rays alone, meaning that the attenuation of X-ray-induced apoptosis by SCF was abolished by the treatment with PD98059. In contrast to this, a combination of SCF with GF109203X or wortmannin attenuated X-ray-induced apoptosis. Treatments with PD98059, GF109203X or wortmannin without X rays had no toxicity for HL60 (data not shown). These observations indicated that the treatment with SCF inhibited the apoptotic process associated with ERK pathways but not PI3-K and PKC pathways.

Since the pharmacological experiments shown in Fig. 1C indicated that the attenuation of X-ray-induced apoptosis by SCF was involved in ERK1/2-regulated survival signals, we examined the expression of phospho-ERK1/2 (active form), and the BCL-2 family was performed as previously described.
ERK1/2 and anti-apoptotic proteins like survivin and BCL-2 in HL60 cells treated with 100 ng/ml of SCF. The incubation of HL60 cells with SCF induced an increase in the expression of phospho-ERK1/2, whereas the expression of total ERK1/2 remained unchanged (first and second panels in Fig. 2). The expression of phospho-ERK1/2 increased within 1 h after SCF treatment, and its activation continued at least up to 8 h. The expression of survivin significantly increased at 4 h after SCF treatment, but the expression of BCL-2 and actin was not influenced by SCF treatment (from third to fifth panels in Fig. 2).

Figures 3A and 3B show the effects of X irradiation on the expression of phospho-ERK1/2 and total ERK1/2 in HL60 without and with SCF. Interestingly, X irradiation alone quickly down-regulated the expression of phospho-ERK1/2 without changing the expression of total ERK1/2 as shown in Fig. 3A, suggesting that X irradiation eliminated ERK-dependent survival signals in HL60 cells. In contrast, combined treatment with X rays and SCF enhanced the expression of phospho-ERK1/2 at 1 h and 2 h after irradiation and it was slightly higher than that with X rays only even at 4 h and 8 h (Fig. 3B). This increase-response of phospho-ERK1/2 induced by SCF or combined treatment of SCF and X rays was significantly inhibited by the treatment of PD98059 (Fig. 3C). The treatment with SCF (lane 2 in Fig. 3D) and X rays+SCF (lane 8 in Fig. 3D) significantly increased the expression of survivin at 8 h, although X irradiation alone (lane 5 in Fig. 3D) had no effect on it. The increased expression of survivin induced by SCF or X rays+SCF was attenuated by PD98059 (lanes 3 and 9 in Fig. 3D), but not by wortmannin.

The tumor cells that express an activating mutation of c-kit acquire a resistance against apoptosis response to chemotherapeutic drugs and ionizing radiation.6–8 This activating mutation of c-kit has been identified at codon 816 (Asp816) in human hematopoietic cell lines.17 Previous studies showed that SCF suppressed apoptosis induced by γ irradiation18,19 and anti-tumor drugs20,21 in vitro and also protected mice22–24 and dogs25 from radiation-induced hematopoietic and intestinal cell death in vivo. In the present study, we also observed that the activation of c-kit by SCF treatment conferred radioresistance to caspase-3-dependent...
Furthermore, the present experiments clearly demonstrated that SCF-induced radioresistance was completely abrogated by the ERK1/2 inhibitor PD98059, and the treatment with SCF significantly enhanced the expression of phospho-ERK1/2, though X irradiation alone quickly decreased its expression. These observations led us to conclude that the acquisition of radioresistance by HL60 caused by the treatment with SCF was due to SCF-induced up-regulation of ERK1/2 signaling pathways. Because survivin\textsuperscript{11}) and BCL-2\textsuperscript{25}) have recently been reported to be candidates for ERK-mediated survival signaling, we examined the expression of these proteins in HL60 cells incubated with SCF. The results obtained from western blots (Fig. 2) showed that the treatment of HL60 cells with SCF increased the expression of survivin but not BCL-2, suggesting that CSF-induced survivin expression was up-regulated by the activation of ERK1/2. In fact, the SCF- or X ray+SCF-induced expression of survivin was abolished by the ERK1/2 inhibitor PD98059 as shown in Fig. 3C.

In conclusion, the activation of c-kit receptor by SCF induced radioresistance through up-regulation of ERK1/2-dependent pathway including survivin expression in HL60 cells. Furthermore, in therapy for c-kit-expressing tumor cells, targeting for survivin with ribozyme, antisense and survivin mutants may be useful to induce apoptosis, reduce tumor growth potential and sensitize cells to X irradiation and chemotherapeutic drugs.

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