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QUARTERLY

TEL/JAK2 tyrosine kinase inhibits DNA repair in the presence of amifostine★✪

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The TEL/JAK2 chromosomal translocation (t(9;12)(p24;p13)) is associated with T cell childhood acute lymphoblastic leukemia. The TEL/JAK2 fusion protein contains the JAK2 catalytic domain and the TEL-specific oligomerization domain. TEL-mediated oligomerization of the TEL/JAK2 proteins results in the constitutive activation of the tyrosine kinase activity. Leukemia cells expressing TEL/JAK2 tyrosine kinase become resistant to anti-neoplastic drugs. Amifostine is a pro-drug which can selectively protect normal tissues against the toxicity of anticancer drugs and radiation. We investigated the effects of amifostine on idarubicin-induced DNA damage and repair in murine pro-B lymphoid BaF3 cells and BaF3-TEL/JAK2-transformed cells using alkaline single cell gel electrophoresis (comet assay). Idarubicin induced DNA damage in both cell types but amifostine reduced its extent in control non-transformed BaF3 cells and enhanced it in TEL/JAK2-transformed cells. The transformed cells did not show measurable DNA repair after exposure to amifostine and idarubicin, but cells treated only with idarubicin were able to recover within a 60-min incubation. Because TEL/JAK2-transformed cells can be considered as model cells

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Abbreviations: FBS, fetal bovine serum; PBS, phosphate-buffered saline.

for certain human leukemias and lymphomas we anticipate an enhancement of idarubicin cytotoxicity by amifostine in these diseases. Moreover, TEL/JAK2 tyrosine kinase might be involved in cellular response to DNA damage. Amifostine could promote apoptosis or lower the threshold for apoptosis induction dependent on TEL/JAK2 activation.

Chromosomal translocations frequently produce fusion genes whose protein products play an essential role in oncogenesis [1]. The TEL/JAK2 fusion proteins result from (t(9;12)(p24;p13)) reciprocal chromosomal translocations and are associated with T-cell acute lymphoblastic leukemia (ALL), atypical chronic myelogenous leukemia (CML), and pre-B-cell ALL [2, 3]. The TEL/JAK2 protein variants may be generated by the fusion of exon 5 of the *TEL* gene to exon 19 of the *JAK2* gene, exon 5 of *TEL* to exon 12 of *JAK2*, or exon 4 of *TEL* to exon 17 of *JAK2* (for details see Fig. 1). This protein contains the catalytic domain of JAK2 juxtaposed to the TEL-specific oligomerization domain. JAK2 is a widely expressed protein tyrosine kinase that associates with the intracellular domains of a number of cytokine receptors and is essential for their functions [4]. TEL is a member of the ETS family of transcription factors and is involved in chromosomal translocations observed in a variety of human leukemias [5, 6]. TEL-mediated oligomerization of TEL/JAK2 results in constitutive activation of JAK2 tyrosine kinase activity. Mutational analysis has revealed that transformation of hematopoietic cells by TEL/ JAK2 requires the oligomerization domain of TEL as well as the kinase activity of JAK2 [7, 8]. TEL/JAK2 fusion tyrosine kinases are localized in the cytoplasm and activate numerous signaling pathways (e.g. STAT5, PI-3k/Akt, ERK2) involved in regulation of growth and apoptosis [9–11]. In addition, recent studies from our laboratories revealed that TEL/JAK2 could induce resistance to DNA damaging agents [12].

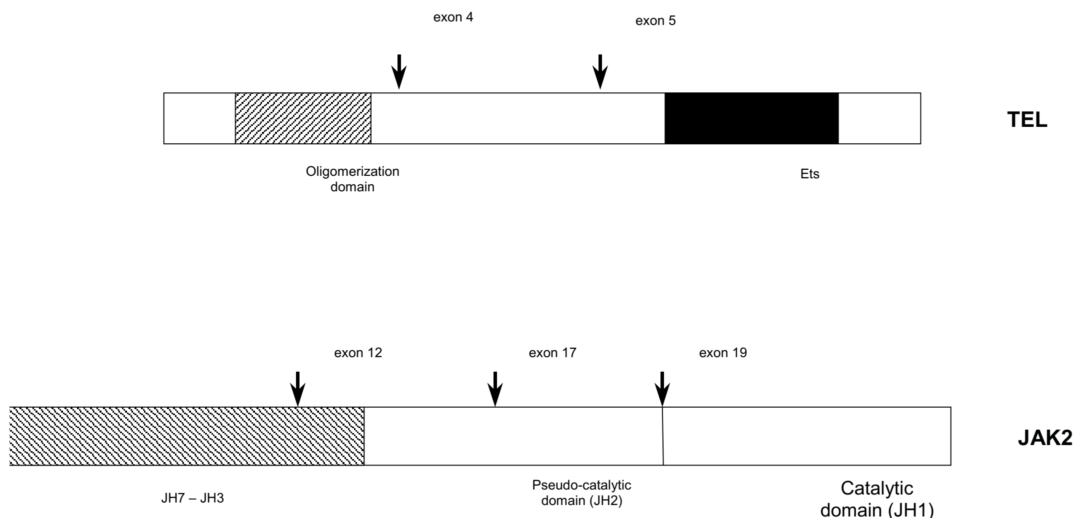

Figure 1. TEL and JAK2 proteins.

Arrows indicate locations of the breakpoints involved in t(9;12) reciprocal translocation.

Idarubicin (4-demethoxy-daunorubicin) (Fig. 2a) is a new synthetic anthracycline anti-cancer agent. Idarubicin complexes with DNA by intercalating between DNA base pairs, thus causing a change of the double helix shape. In addition, this drug stabilizes topoisomerase II, causing double-strand DNA breaks [13] and generates free radicals [14].

Chemical radioprotectors have been developed to prevent drug- and radiation-induced

toxicity in normal cells. The most prominent example of them is amifostine (s-2[3-aminopropylamino]ethyl phosphothioic acid; Fig. 2b), a drug that is commonly approved for use in the treatment of cancer. Amifostine is a pro-drug converted by alkaline phosphatase to its active, dephosphorylated form WR-1065, which can cross the cell membrane by both passive and active diffusion [15]. WR-1065 is a potent scavenger of reactive oxygen species and there is convincing evidence that it reduces the extent of DNA damage caused by free radicals both *in vitro* and in intact cells [16]. However, recent investigations have identified a number of additional amifostine-induced effects that could modify cellular response to anticancer drugs. These include an inhibition of apoptosis [17], alteration of gene expression (e.g. *c-myc*, thymidine kinase gene) [18] and modification of enzyme activity (e.g. matrix metalloproteinases, topoisomerase II-*a*) in normal cells [19]. Interestingly, it has been reported that amifostine might exert different biological effects in normal *versus* leukemia cells [20–22].

The present study was undertaken to examine the effect of amifostine on idarubicin-induced genotoxicity in normal cells (murine pro-B lymphoid cell line BaF3) and leukemic BaF3-TEL/JAK2-transformed cells. The extent of DNA damage and kinetics of DNA repair were assessed using alkaline single cell gel electrophoresis (comet assay).

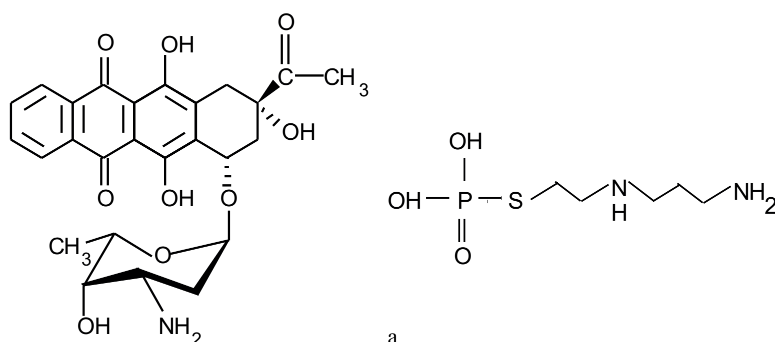

Figure 2. Chemical structure of idarubicin (a) and amifostine (b).

MATERIALS AND METHODS

Chemicals. Idarubicin was obtained from Pharmacia Upjohn (Milan, Italy). Amifostine, Tris, RPMI 1640 medium without glutamine, fetal bovine serum (FBS), agarose, low melting point agarose, phosphate-buffered saline (PBS) and DAPI (4′,6-diamidino-2-phenylindole) were obtained from Sigma Chemicals Co. (St. Louis, MO, U.S.A.).

Cells. Murine growth factor-dependent pro-B lymphoid cell line BaF3 and BaF3-TEL/JAK2 transformed clone were obtained from Dr. Gary Gilliland (Brigham and Women's Hospital, Boston, MA, U.S.A.). Cell lines were maintained in RPMI 1640 supplemented with 10% FBS and 15% WEHI-conditioned medium (growth medium). The viability of the cells was measured by trypan blue exclusion staining and was about 99%. The final concentration of the cells was adjusted to $1-3 \times 10^5$ cells/ml by adding the growth medium.

DNA repair. To examine DNA repair, BaF3 and BaF3-TEL/JAK2 cells (1.5×10^6/ml) were incubated with 14 mM amifostine for 15 min at 37°C followed by treatment with 0.03, 0.3 and 1.0 μM idarubicin for 1 h at 37°C. Negative control groups were incubated without the drugs. After treatment cells were washed and re-suspended in drug-free growth medium. Aliquots of cell suspensions were harvested immediately (time 0) and after 30, 60 and 120 min, and placed on ice to stop the re-

pair reactions. Cells exposed to 10 μM hydrogen peroxide for 5 min at 4°C served as positive control.

Comet assay. The comet assay was performed under alkaline conditions essentially according to the procedure of Singh *et al.* [23] with some modification [24] as described previously [25]. A freshly prepared cell suspension in 0.75% low melting point agarose dissolved in PBS was placed onto microscope slides pre-coated with 0.5% normal melting agarose. The cells were then lysed for 1 h at 4°C in a buffer consisting of 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM Tris, pH 10. After the lysis, the slides were placed in an electrophoresis unit, DNA was allowed to unwind for 40 min in an electrophoretic solution consisting of 300 mM NaOH, 1 mM EDTA, pH >13. Electrophoresis was conducted at 4°C (the temperature of the running buffer not exceeding 12°C) for 30 min at electric field strength 0.73 V/cm (30 mA). The slides were then neutralized with 0.4 M Tris, pH 7.5, stained with 2 μg/ml DAPI and covered with cover slips. To prevent additional DNA damage all steps were conducted under dimmed light or in the dark.

Comet analysis. The slides were examined at 200 × magnification in an Eclipse fluorescence microscope (Nikon, Japan) attached to a COHU 4910 video camera (Cohu, Inc., San Diego, CA, U.S.A.) equipped with a UV filter block consisting of an excitation filter (359 nm) and a barrier filter (461 nm) and connected to a personal computer-based image analysis system, Lucia-Comet v. 4.51 (Laboratory Imaging, Czech Republic). Fifty images were randomly selected for each sample and the comet tail moment (a product of fraction of DNA in tail and tail length) was measured. Two parallel tests with aliquots of the same sample of cells were performed for a total of 100 cells and the mean comet tail moment was calculated. The comet tail moment is positively correlated with the level of DNA breakage in a cell [26]. The mean value of the tail moment in a particular sample was taken as an indicator of DNA damage.

Statistical analysis. All the values in this study were expressed as mean ± S.E.M. pooled results of two parallel tests were considered. If no significant differences between variations were found, as assessed by Snedecor-Fisher test, the differences between means were evaluated by applying Student's *t*-test. The dates were analyzed using the STATISTICA (StatSoft, Tulsa, OK, U.S.A.) statistical package.

RESULTS

DNA damage

An analysis of the comet tail moment of cells after treatment with amifostine and/or idarubicin is shown in Fig. 3. Preincubation of

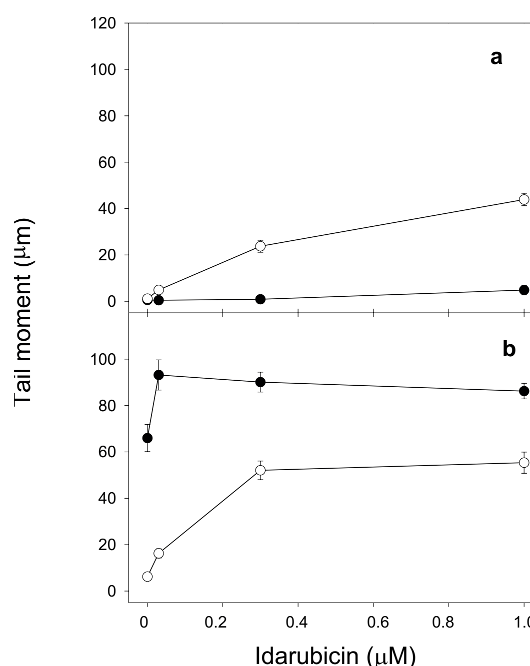

Figure 3. Mean comet tail moment of (a) BaF3 cells and (b) BaF3-TEL/JAK2-transformed cells.

Cells were exposed to idarubicin (○) or preincubated for 15 min with 14 mM amifostine and exposed for 1 h at 37°C to idarubicin (●). Results represent mean ± S.E.M. from 100 cells. Error bars denote S.E.M.

BaF3 cells with 14 mM amifostine significantly reduced the tail moment after incubation with idarubicin (Fig. 3a) ($P < 0.001$). BaF3-TEL/JAK2-transformed cells preincubated with amifostine displayed significantly increased tail moment after exposure to idarubicin (Fig. 3b) ($P < 0.001$). We observed also comets which can be attributed to apoptotic cells (Fig. 4). Interestingly, amifostine by itself caused DNA damage in BaF3-TEL/JAK2 cells but not in BaF3 cells (Fig. 3).

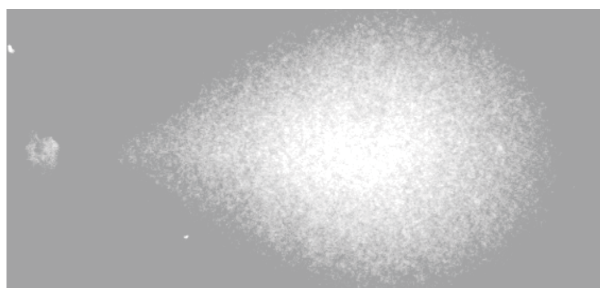

Figure 4. Typical apoptotic comet of BaF3-TEL/JAK2-transformed cell preincubated for 15 min with 14 mM amifostine and exposed for 1 h at 37°C to idarubicin.

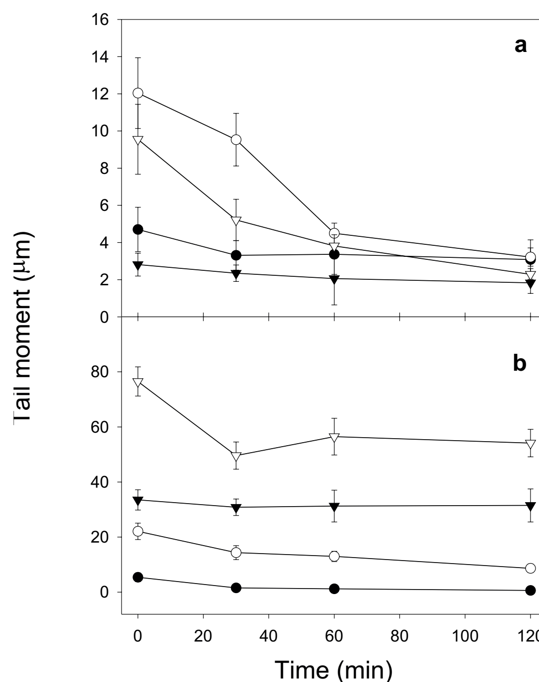

Figure 5. Time course of the repair of DNA damage in (a) BaF3 control cells and (b) BaF3-TEL/JAK2-transformed cells.

Cells were treated with amifostine (▼), idarubicin (○) and amifostine followed by idarubicin (▽), or were left untreated (●). Results represent mean ±S.E.M. from 100 cells.

DNA repair

Reparation of the damaged DNA was measured by analyzing the comet tail moment at different time-points after idarubicin treatment (Fig. 5). Pretreatment of BaF3 cells with amifostine accelerated DNA repair (Fig. 5a). On the other hand, BaF3-TEL/JAK2 cells pretreated with amifostine displayed slower kinetics of DNA repair as indicated by detection of substantial comet tail moments even after 120 min of incubation time (Fig. 5b).

DISCUSSION

Anti-neoplastic drugs usually have narrow therapeutic index, i.e. the difference between the toxic and the effective dose. Side effects of these drugs include tissue toxicity and secondary malignancies, probably due to the induction of genetic instability. Therefore, selective protection of normal *versus* tumor tissues seems to be an important goal.

Amifostine has been reported to be a selective cytoprotective compound [27–29]. The active form of amifostine, WR-1065, might be involved in two types of general cell responses to DNA damage induced by idarubicin: a reduction of the extent of initial damage by its radical scavenging ability and an enhancement of the effectiveness of repair processes by prolongation of G_2/M cell cycle phase [30].

In the present study a protective effect of amifostine against idarubicin-induced genotoxicity in normal (BaF3) as well as model leukemic (BaF3-TEL/JAK2-transformed) cells was examined by the comet assay. We show that pretreatment with amifostine reduced the extent of DNA damage caused by idarubicin in BaF3 cells. In contrast, BaF3-TEL/

JAK2 cells exposed to amifostine subsequently treated with idarubicin did not show measurable DNA repair. Our results are in agreement with other reports indicating that amifostine enhanced cyclophosphamide and mafosfamide cytotoxicity in myeloid and lymphoid leukemia cells [20, 21].

The mechanism of the selective genotoxic effect of amifostine in BaF3-TEL/JAK2 cells is not known. However, in agreement with this observation amifostine suppressed the proliferation and clonogenic growth of leukemic cells from patients with acute myeloid leukemia or chronic myeloid leukemia [22].

Taken together, our results implicate amifostine in protection of normal cells against the DNA damaging effects of idarubicin. In addition, amifostine seems to inhibit DNA repair and increase the genotoxic effect of idarubicin in leukemia cells. Thus, amifostine may work as a compound increasing the therapeutic index of anti-neoplastic drugs.

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