Enhanced Expression of DNA Topoisomerase II by Recombinant Human Granulocyte Colony-stimulating Factor in Human Leukemia Cells

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ABSTRACT

The effect of recombinant human granulocyte colony-stimulating factor (G-CSF) on DNA topoisomerase II (topo II) expression was studied in two human acute myelogenous leukemia cell lines, NKM-1 and NOMO-1, which express G-CSF receptor and proliferate in response to exogenous G-CSF. Northern blot analysis revealed that the level of topo II mRNA in 16-h stimulated cells in serum-free medium with G-CSF (10 ng/ml) was approximately 2-fold higher than that in cells without G-CSF. Enhanced topo II mRNA expression was detectable within 3 h after the addition of G-CSF. Topo II activity in crude nuclear extracts from 16-h G-CSF-stimulated cells was also found to be approximately 2-fold greater than that from unstimulated cells. According to in vitro cytotoxic assay, the sensitivity of G-CSF-stimulated cells to intercalating (daunorubicin) and nonintercalating (etoposide) topo II-targeting drugs increased significantly, whereas no enhancement of sensitivity was observed with an alkylating agent (4-hydroperoxycyclophosphamide). The augmented drug sensitivity observed was not due to the increased level of drug transport, as suggested by the similar extent of [3H]etoposide uptake between G-CSF-stimulated and unstimulated cells. By measuring the topo II mRNA and the cytotoxicity of the above mentioned drugs, we obtained essentially the same results in G-CSF-responsive leukemia cells isolated from three acute myeloid leukemia patients, as observed in the cultured cell lines. These findings strongly suggest that the sensitivity to "topo II-targeting drugs" could be augmented by exogenous G-CSF through elevated topo II activity in G-CSF-responsive leukemia cells.

INTRODUCTION

One of the major objects of research on leukemia therapy is the identification of critical biochemical targets that mediate the ability of chemotherapeutic agents to kill leukemia cells without disturbing normal cell function. The cellular enzyme topo II is an attractive candidate for such a target, because it has been implicated in DNA replication, transcription, and repair (1) and identified as an intracellular target of a number of clinically important antitumor drugs (2-4). The cellular levels of this enzyme have been shown to correlate well with the extent of drug sensitivity (5-8), and thus reduced amounts of topo II lead to resistance in treatment with topo II-targeting drugs (7-13). Therefore, it is postulated that the magnitude of drug-induced, topo II-mediated cytotoxicity could be modulated by changing cellular topo II levels. Others have reported stimulation of topo II activity by some kinds of cytokines or anti-metabolites (14-18). The strategy for effectively regulating topo II on human leukemia cells, however, has not been fully explored. Recently, molecular cloning and functional analysis of CSFs have revealed their essential roles in normal hematopoiesis, and a large number of clinical studies have provided substantial data for in vivo effectiveness. It is also widely recognized that the growth of human acute myelogenous leukemia cells is stimulated by various CSFs (19-23). In this context, the present study was designed to investigate the effect of G-CSF on topo II expression and sensitivity to various antitumor drugs in human leukemia cells proliferating in response to G-CSF. We demonstrate the feasibility of altering sensitivity of these neoplastic cells to topo II-mediating antitumor drugs.

MATERIALS AND METHODS

Biological Reagents. Recombinant human G-CSF (KRN 8601) produced in Escherichia coli was obtained from KRN Brewery Co., (Tokyo, Japan). DNR was purchased from Meiji Seika K.K. (Tokyo, Japan) and VP-16 from Bristol-Myers K.K. (Tokyo, Japan). 4-HC was a gift from Shionogi Seiyaku Co., Ltd. (Osaka, Japan).

Cell Lines and Cell Cultures. Two myeloid cell lines, NKM-1 (M2) and NOMO-1 (M5), have been established in our laboratory and maintained in RPMI 1640 supplemented with 10% fetal calf serum for >2 years (24). Both of them express G-CSF receptors and are stimulated to proliferate by the addition of G-CSF in culture. 

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Preparation of Crude Nuclear Extracts. Crude nuclear extracts were prepared by a slight modification of a previously published procedure (28). Cells incubated for 16 h were washed in ice-cold phosphate-buffered saline (0.14 M NaCl-2.7 mM KCl-6.4 mM Na2HPO4-1.5 mM KH2PO4, pH 7.4) and suspended at a density of 1 × 10^6 cells/ml in solution A (5 mM KH2PO4, pH 7.5-1 mM MgCl2-0.005% Triton X-100-fresh 1 mM phenylmethanesulfonyl fluoride). Twenty min later, they were lysed by addition of an equal volume of solution B (2 mM NaCl-100 mM Tris-HCl, pH 7.6, 1 mM DTT-0.005% Triton X-100-fresh 1 mM phenylmethanesulfonyl fluoride). After standing for 20 min, an equal volume of solution C (18% polyethylene glycol-1 mM NaCl-1 mM DTT) was added slowly to precipitate DNA. After 30 min on ice, the mixture was centrifuged at 17,000 × g for 20 min and the supernatant was saved. Extract protein concentrations from G-CSF-stimulated and -unstimulated cells were determined by the method of Bradford (29) and adjusted to equivalence.

Decatenation Assay of Kinetoplast DNA. Form 1 kDNA was isolated from the mitochondria of Crithidia fasciculata according to the established procedure (30). The quantitative decatenation assay was a slight modification of earlier reports (28, 31). Serial dilutions of extract were incubated with 0.28 μg of kDNA in a final volume of 50 μl at 30°C for 30 min. The reaction was terminated by the addition of 5 μl of a mixture of 5% sodium dodecyl sulfate, 50% glycerol, and 0.5% bromphenol blue. The samples were electrophoresed on 1% agarose gels and the density of these bands on the negatives was measured by a densitometric scanner, and the percentage of decatenation was calculated.

Cytotoxicity Assay. DNR was used as an intercalator, VP-16 as a nonintercalator, and 4-HC as a control agent. Cultured cells pretreated with various concentrations of [3H]VP-16 (400 mCi/mmol; Moravek Biochemicals, Brea, CA) for 1 h at 37°C. Cell survival curves were corrected for the plating efficiency of cells which had been incubated with the preincubation medium alone. Each experiment was performed at least three times, and the data were analyzed statistically for significance (Student's t test). The effect of G-CSF on cytotoxicity was expressed quantitatively as the dose required to kill 50% of the cells.

Drug Uptake Study. Prior to drug uptake assay (33-35), NKM-1 and NOMO-1 cells were incubated in RPMI 1640 with or without G-CSF for 16 h at a concentration of 3 × 10^6 cells/ml or patients' samples at a concentration of 2 × 10^6 cells/ml were subsequently incubated with various concentrations of freshly dissolved drugs for 2 h at 37°C. Cells were then washed twice and dispersed equally into 96-well flat-bottomed microplates in triplicate. After incubation for 24 h, the viable cells were measured by a colorimetric assay using tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (32). Cell survival curves were corrected for the plating efficiency of cells which had been incubated with the preincubation medium alone. Each experiment was performed at least three times, and the data were analyzed statistically for significance (Student's t test). The effect of G-CSF on cytotoxicity was expressed quantitatively as the dose required to kill 50% of the cells.

RESULTS

Topo II mRNA Expression. Total RNA isolated from various cell lines incubated with or without G-CSF for 16 h was investigated by Northern blot analysis. Representative data are shown in Fig. 1A. Densitometric scanning was used to compare the intensity of 6.2-kilobase topo II message relative to that of HLA-B7 message; this ratio was 3.56 in G-CSF-stimulated NKM-1 cells.
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NKM-1 cells, 1.79 in control NKM-1 cells, 3.16 in G-CSF-stimulated NOMO-1 cells, and 1.93 in control NOMO-1 cells. Hence, there was approximately a 1.99-fold and a 1.64-fold increase in intensity of topo II message in G-CSF-stimulated NKM-1 and NOMO-1, respectively. From three individual experiments of NKM-1 cells or NOMO-1 cells, 2.19 ± 0.44-fold (mean ± SD) or 1.65 ± 0.31-fold increases were observed, respectively, and the difference in each case was statistically significant (P < 0.05). However, no differences between G-CSF-stimulated and -unstimulated cells were observed in either HL-60 or MEG-01. In a similar fashion, increased intensity of topo II messages was observed in fresh G-CSF-responsive leukemia cells (Patient 1, 4.7-fold; Patient 2, 2.6-fold; Patient 3, 3.3-fold), whereas there was no alteration in G-CSF-nonresponsive leukemia cells (Patient 4) (Fig. 1B). Although a direct comparison seemed to be difficult, the enhanced effect of G-CSF in freshly obtained samples was much larger than in cultured cells.

Time-course analysis of topo II mRNA expression in NKM-1 cells revealed that G-CSF caused a rapid induction of the mRNA within 90 min (1.9-fold increase compared to the basal level) and reached a 2.5-fold increase at 6 h (Fig. 1C). The expression of topo II mRNA of control cultures was not changed during the observation periods.

**Topo II Catalytic Activity.** To evaluate the effect of G-CSF on the enzyme activity of topo II, a quantitative decatenation assay was carried out. Equivalent amounts of protein from crude nuclear extracts of G-CSF-stimulated and -unstimulated cells were incubated with kDNA. As shown in Fig. 2, approximately 2-fold greater catalytic activity was observed in extracts from both NKM-1 and NOMO-1 cell lines stimulated by G-CSF when compared to those from unstimulated cells. The amount of nuclear extract proteins required to decatenate 50% of the kDNA was reduced from 0.19 to 0.09 μg in NKM-1 cells by the addition of G-CSF. Similarly, it was altered from 0.24 to 0.13 μg in NOMO-1 cells.

Effect of G-CSF on Drug-mediated Cytotoxicity. Fig. 3 demonstrates the cytotoxic effects of DNR, VP-16, and 4-HC in
NKM-1 and NOMO-1. Both cell lines pretreated with G-CSF showed an increased sensitivity to topo II poisons, DNR and VP-16, but not to 4-HC. There were statistical significances between G-CSF-stimulated cells and -unstimulated cells at a serial concentration of both DNR and VP-16 (P < 0.05, t test). Each of the NKM-1 and NOMO-1 cell lines pretreated with G-CSF demonstrated a simple exponential DNR dose-dependent survival curve (36) with doses required to kill 50% of the cells of 0.5 ± 0.1 or 3.0 ± 0.3 μM, respectively, in contrast to 0.75 ± 0.1 or 4.2 ± 0.4 μM for control NKM-1 or NOMO-1 cell (1.5- or 1.4-fold), respectively. Similarly, their sensitivity to VP-16 also increased approximately 2.5-fold or 1.3-fold, respectively, by G-CSF. With respect to 4-HC, reduced cytotoxicity was observed in G-CSF-pretreated cells, whereas NKM-1 showed no difference in drug sensitivity. Also, in fresh acute leukemia cells from Patient 3, VP-16-induced cytotoxicity at the concentration of 5 μM increased from 17.33 ± 5.86 to 34.33 ± 5.51% (P = 0.031) after pretreatment with G-CSF, and in a similar fashion, DNR-induced cytotoxicity at the concentration of 0.1 μM rose remarkably from 1.33 ± 2.31 to 14.00 ± 6.93% (P = 0.040).

G-CSF Effect on Drug Accumulation. No differences in drug uptake were observed between G-CSF-treated cells and -untreated cells at all drug concentrations examined (Fig. 4). The same result was obtained in NOMO-1 cells (data not shown). These results suggest that the increased cytotoxicity of VP-16 by G-CSF was not due to the difference in cellular accumulation of VP-16.

DISCUSSION

In the past decade, topo II has emerged as a critical intracellular target of several clinically important chemotherapeutic agents including the nonintercalative epipodophillotoxins as well as diverse intercalative compounds. Several factors appear to influence the magnitude of topo II-mediated cytotoxicity induced by chemotherapeutic agents: (a) the proliferative state of the cell, (b) the cell cycle state, and (c) the chromatin conformation. Among many reports on the regulation of topo II expression (18, 28, 37-41), Hsiang et al. (40) demonstrated that topo II levels in transformed cells such as HeLa cells and L1210 cells were maintained at high basal levels and were less sensitive to growth conditions than those in untransformed cells. Furthermore, the topo II level in HeLa cells remained constant throughout the late G1, S, G2, and M phases of the cell cycle. Therefore, in the present study, in order to obtain more significant differences in topo II levels, as well as to study an essential effect of G-CSF, we have designed serum-free culture conditions in which G0/G1 cells were relatively enriched and kept alive during the experimental period. In the presence of G-CSF, at least a 1.5-fold increase in topo II mRNA was observed in NKM-1 and NOMO-1 cell lines under these conditions. Furthermore, the effect of G-CSF on topo II mRNA expression was observed at the latest within 3 h after the addition of G-CSF while the percentage of S-phase cells remained unchanged. It should also be noted that a more prominent increase in topo II mRNA was observed in freshly obtained leukemia cells, most of which probably remained in the G0/G1 phase of the cell cycle. These findings support the hypothesis that the topo II expression occurs in the early G1 phase of the cell cycle.

Since G-CSF has been reported to exert a proliferative effect on human myeloid leukemia cells, the clinical application of G-CSF has been limited to patients with non-myeloid leukemia. Nevertheless, Miyauchi et al. (42) offered the explanation that the effectiveness of S-phase-specific agents such as 1-β-D-arabinofuranosylcytosine was increased by stimulating myeloid leukemia blasts into the active cell cycle with granulocyte-macrophage colony-stimulating factor and G-CSF (43). However, there are reports that the biological state of the target enzyme of chemotherapeutic agents is altered by exogenous CSFs. This is the first report of a successful enhancement of topo II expression by one of the CSFs.

Changes in topo II were proved to contribute to some forms of resistance to topo II poisons in light of the well-collaborated correlation between a decreased level of topo II activity and drug resistance (12, 13, 33). Therefore, agents elevating topo II activity of tumor cells may be expected to be a new chemotherapeutic modality. We have provided evidence that the drug sensitivity of G-CSF-treated myeloid leukemia cells specifically increased against DNR and VP-16, in parallel with the increment of the topo II level. Clearly, the increased sensitivity was not due to changes in intracellular accumulation of these drugs. It is generally accepted that such effects of G-CSF would be due to an elevated intracellular topo II level, because many investigators have reported a strong correlation between the amount of topo II and the cytotoxic effect of topo II inhibitors. These findings point the way to future clinical studies. Use of G-CSF prior to use of topo II inhibitors may be applied not only to cancer chemotherapy protocols but also to the conditioning regimens of bone marrow transplantation in order to obtain a higher efficacy of tumor cell death without escalating the adverse effects on nonhematopoietic cells. It may be also feasible to combine administration of G-CSF and topo II poisons to effectively reverse some forms of drug resistance mediated by quantitative changes in topo II. However, there are several potential problems in translating our in vitro observations into a clinical trial. First, the degree of leukemic burden may have a negative influence on leukemic cell proliferation. In addition, it is possible that G-CSF might be acting on later stage cells rather than the leukemic progenitors. Finally, it is likely that G-CSF increases the sensitivity of normal myeloid progenitor cells to topo II poisons resulting in prolonged myelosuppression. Therefore, a combined administration trial of G-CSF and topo II poisons in the treatment of G-CSF-responsible myeloid leukemias should be carefully designed to evaluate the toxicity and efficacy of this clinical approach. Further studies in vivo are to be executed to examine the clinical usefulness of G-CSF in the treatment of myeloid leukemias.

![Fig. 4. Cellular accumulation of [H]VP-16 of control (●) and G-CSF-stimulated NKM-1 cells (○). The two curves are not significantly different and are displayed with a common slope. Points, representative of two separate experiments; SD values (bars) were consistently <10% of the means.](image-url)
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