Cyclic AMP-dependent Protein Kinase Type I Is Involved in Hypersensitivity of Human Breast Cells to Topoisomerase II Inhibitors

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ABSTRACT

Topoisomerase II (Topo II) is an essential enzyme that catalyzes the breakage of double-strand DNA and is the target of several effective anticancer drugs, including the epipodophyllotoxins. The regulatory subunits of the cyclic AMP-dependent protein kinase are differentially expressed in normal and cancer cells. The R1α subunit is overexpressed in cells transformed by transforming growth factor-α (TGF-α) or Ha-ras oncogene. It has been shown that murine cells transformed by Ha-ras become hypersensitive to Topo II-targeting anticancer drugs. In this report we have tested whether any correlation exists between the expression of R1α protein and cellular sensitivity to Topo II-targeting drugs. Normal human breast MCF-10A cells and their derivatives overexpressing TGF-α, Ha-ras, or the different protein kinase subunits were treated with either Topo II inhibitors, such as etoposide, teniposide, or amsoacin, or with drugs which act independently of Topo II, such as bleomycin. Here we show that MCF-10A/TGF-α and MCF-10A Ha-ras cells overexpress the R1α protein and become hypersensitive to epipodophyllotoxins and amsoacin but not to bleomycin. Direct introduction of the R1α gene into MCF-10A induces hypersensitivity to Topo II inhibitor drugs. In contrast, the overexpression of the other protein kinase subunits, R1β or Cα, does not modify the drug sensitivity of MCF-10A cells. No differences in the mRNA/protein content or in the activity of Topo II were found between hypersensitive cells and parental MCF-10A cells, suggesting that R1α may influence drug sensitivity via modulation of events downstream of the Topo II-DNA cleavable complex.

INTRODUCTION

Topo II is an essential nuclear enzyme which catalyzes the concerted breakage of double-stranded DNA and passage of a second duplex through the break (1–3). Topo II is a constituent of the mitotic chromosome scaffold (4) and has an essential role in the segregation of replicated DNA at mitosis (5–7). Several of the most effective anticancer drugs, including the epipodophyllotoxins, etoposide and teniposide, exert their cytotoxicity via the stabilization of a covalent DNA–Topo II complex termed the cleavable complex, which is a normal transient intermediate in the Topo II catalytic cycle (8, 9). In mammalian cells, there are two closely related isoforms of Topo II, designated α and β, which are encoded on different chromosomes (10, 11) and are apparently differentially regulated (12).

cAMP acts in mammalian cells by binding to either of two distinct classes of the cAMP-dependent PKA, defined as type I (PKAI) and type II (PKAII), respectively (13). The isoforms share common catalytic subunits but differ in their cAMP-binding regulatory subunits. Two different R isoforms have been described for PKAI, termed R1α and R1β, and two for PKAII, termed R1IIα and R1IIβ, respectively (13). Among the different C isoforms, Cβ is the most widely studied in eukaryotic cells. Differential expression of PKA regulatory subunits has been correlated with cell differentiation and neoplastic transformation (14). Preferential expression of R1IIβ has been observed in normal tissues or in growth-arrested cells, while enhanced levels of R1α are generally found in tumor cells or in normal cells following exposure to mitogenic stimuli (14–17).

We have previously shown a functional link between expression of oncogenes and changes in the R1α protein levels. NIH 3T3 fibroblasts or normal rat kidney fibroblasts transformed by the Ki-ras oncogene synthesize increased levels of R1α protein (18, 19). Transformation of either normal rat kidney cells or mouse mammary epithelial NOG-8 cells with the TGF-α gene also led to an increase in R1α levels (18, 19). Selective down-regulation of R1α levels by specific agents such as site-selective cAMP analogues (14) was accomplished in all of the above cell lines by both an inhibition of Ki-ras and TGF-α expression, and an inhibition of cellular growth (18, 19). NIH 3T3 cells transformed by the ras oncogene have been shown to contain elevated levels of Topo IIα protein and are correspondingly more sensitive to Topo II-targeting drugs than are the parental cells (20).

In this study we have investigated (a) whether increased sensitivity to Topo II-targeting drugs is observed in human...
epithelial cells transformed by the ras or TGF-α genes and (b) whether a correlation exists between hypersensitivity to Topo II-targeting drugs and the expression of the R1α gene. For this purpose we have used the spontaneously immortalized, non-transformed human mammary epithelial cell line, MCF-10A (21). We show that overexpression of the R1α gene is accompanied by cellular hypersensitivity to Topo II inhibitors.

MATERIALS AND METHODS

Cell Cultures. MCF-10A cells and their derivatives were grown in a 1:1 (v/v) DMEM and Ham’s F12 mixture supplemented with 5% heat-inactivated horse serum, 20 mm HEPES (pH 7.4), 4 mm glutamine (ICN Biomedicals, Costa Mesa, CA), 0.5 μg/ml hydrocortisone (Sigma Chemical Co., St. Louis, MO), 10 ng/ml epidermal growth factor, and 10 μg/ml insulin (Collaborative Research, Bedford, MA) in a humified atmosphere of 95% air-5% CO₂ at 37°C. MCF-10A ras cells are a clonal cell line generated by cotransfection of MCF-10A cells with an expression vector plasmid containing the human activated c-Ha-ras protooncogene and an expression vector plasmid containing the neomycin resistance gene (22). MCF-10A neo cells are MCF-10A cells transfected with the plasmid containing only the neomycin resistance gene. MCF-10A TGF-α, MCF-10A R1α, MCF-10A R1Iβ, and MCF-10A Ca cells were generated by infection with recombinant amphotropic retroviral vectors containing the neomycin resistance gene along with the human TGF-α or the human R1α, R1Iβ, and Ca genes, respectively. Expression of these genes was under the transcriptional control of the heavy metal-inducible mouse metallothionin-I promoter (22, 23). MCF-10A TGF-α, MCF-10A R1α, MCF-10A R1Iβ, and MCF-10A Ca cells were continuously grown in the presence of 1 μM CdCl₂ to maximally induce the expression of the metallothionin-I promoter. We have previously shown that this concentration of CdCl₂ is not toxic for MCF-10A cells and does not affect their growth (22).

Monolayer Growth and Drug Sensitivity Measurement. Cells (10⁴) were plated in 96-well clusters (Becton Dickinson Italia, Milan, Italy) and treated with different concentrations of etoposide, teniposide, amsacrine, or bleomycin (Sigma) for 1 h. Concentrations of CdCl₂ are not toxic for MCF-10A cells and does not affect their growth (22).

RESULTS

We recently generated transformed clones of MCF-10A cells via the overexpression of either a human point-mutated c-Ha-ras gene or the human TGF-α gene (22). To determine whether overexpression of Ha-ras and TGF-α in MCF-10A cells induced hypersensitivity to Topo II-targeting drugs, the effect of etoposide, teniposide, or amsacrine on the cell growth of these transformed cells as compared to control MCF-10A neo cells was analyzed. MCF-10A ras and MCF-10A TGF-α cells were more sensitive to growth inhibition by the epipodophyllotoxins, etoposide and teniposide, and by amsacrine than were MCF-10A neo cells, exhibiting a 2- to 4-fold lower IC₅₀ (Fig. 1) and data not shown). In contrast, MCF-10A ras and MCF-10A TGF-α cells were more resistant than MCF-10A neo cells to bleomycin, a drug which does not act through Topo II (Fig. 1).
neo cells only a low level of the 49-kDa Rlα species was detected, MCF-10A ras and MCF-10A TGF-α cells showed a 20- and a 10-fold increase in Rlα protein levels, respectively (Fig. 1). No differences were observed in the growth pattern and the expression of Rlα protein in MCF-10A neo cells as compared to parental MCF-10A cells (data not shown).

To directly assess whether the overexpression of Rlα protein may be implicated in the acquired hypersensitivity of these cells to Topo II drugs, MCF-10A cells were infected with a series of retroviral vectors encoding the Rlα, the Rlβ, or the Ca subunit of PKA (23). Western blotting analysis of cell extracts from MCF-10A neo, MCF-10A Rlα, MCF-10 Rlβ, and MCF-10A Ca cells using the antihuman Rlα monoclonal antibody showed that while in MCF-10 Rlβ and MCF-10A Ca cells the expression of Rlα protein was similar to that of MCF-10A neo cells, a 10- to 20-fold increase in the level of Rlα protein was observed in MCF-10A Rlα as compared to parental MCF-10A cells (Fig. 2).

The infected cells were then tested for sensitivity to cytotoxic drugs. MCF-10A Rlα cells exhibited hypersensitivity to etoposide and teniposide with approximately a 3-fold and 2-fold lower I_{50} value, respectively, than MCF-10A neo, MCF-10A Rlβ, or MCF-10A Ca cells (Fig. 2). MCF-10 Rlα also showed a hypersensitivity to amsacrine (2-fold), another Topo II-targeting drug (data not shown). This observed hypersensitivity to Topo II-targeting drugs was not a result of a change in the cell cycle distribution of the cells overexpressing Rlα protein (data not shown). MCF-10A Rlβ and MCF-10A Ca cells did not show any difference in their sensitivity to etoposide, teniposide, or amsacrine as compared to MCF-10A neo cells. Interestingly, in the case of bleomycin, enhanced resistance rather than sensitivity was observed for MCF-10A Rlα cells as compared to MCF-10A neo cells, which paralleled the results obtained in MCF-10A cells transformed by ras or TGF-α. We analyzed the effect of etoposide and bleomycin on the colony-forming ability of these cells using a clonogenic assay. MCF-10A Rlα and MCF-10A ras cells were each approximately 2–4 fold more sensitive than MCF-10A neo cells to etoposide. In contrast, doses of bleomycin which produced approximately 50% survival with MCF-10A neo cells, were not toxic to MCF-10A Rlα and MCF-10A ras cells (Fig. 3).
Fig. 2  Differential effect of etoposide, teniposide, and bleomycin and Western blotting analysis of Rla expression in MCF-10A cells infected with different PKA subunits. MCF-10A neo, MCF-10A Rla, MCF-10A RIIß, and MCF-10A Cu cells were treated with different drugs 24 h after plating and cell survival was determined by the MTT assay as described in “Materials and Methods.” Data are from a single experiment representative of three independent assays showing similar results. Cell extracts from MCF-10A neo (Lane 1), MCF-10A RIIß (Lane 2), MCF-10A Cu (Lane 3), and MCF-10A Ri (Lane 4) cells were tested with a monoclonal antibody directed against the 49-kDa product of the human Rla subunit of PKAl as described in “Materials and Methods.”

Fig. 3  Effect of etoposide and bleomycin on MCF-10A, MCF-10A Rla, and MCF-10A ras colony formation. Clonogenic survival of cells following exposure to etoposide or bleomycin. Cells were treated as described in “Materials and Methods.” Data represent the average ± SD of two different experiments.
It has been shown that hypersensitivity to Topo II-targeting drugs may be correlated to the increased levels of Topo II protein (24). To evaluate whether a similar phenomenon had occurred in the MCF-10A cells overexpressing R1α protein, the levels of mRNA expression for the two human Topo II isozymes (α and β) were determined by the RNase protection assay. Fig. 4 shows that Topo IIα and Topo IIβ mRNA levels were similar in MCF-10A R1α and MCF-10A parental cells. Simultaneous measurement of Topo IIα protein levels and DNA content during the cell cycle by flow cytometry indicated that Topo IIα protein levels were not altered in MCF-10A ras or MCF-10A R1α cells (Fig. 4). Similar results were also obtained with Western blotting analysis using a polyclonal antibody directed against the human Topo IIα protein (Ref. 24; data not shown).

To determine whether the differential sensitivity to Topo II drugs was due to an increase in Topo II enzyme activity in MCF-10A R1α cells, a decatenation assay using nuclear protein extracts was performed. There was no significant difference in the level of Topo II activity in extracts from MCF-10A neo and MCF-10A R1α cells (Fig. 5).

**DISCUSSION**

Overexpression of the R1α subunit of PKA has been shown to correlate both with increased cell proliferation and with neoplastic transformation, and has therefore been proposed as a novel target for cancer therapy (14, 15, 26, 27). The nuclear enzyme Topo II may also play a key role in regulating cell proliferation and represents a target for several currently used and effective anticancer drugs. Although extensively studied,
decreases with cell confluency (12, 33). MCF-IOA cells over-

does not target Topo II. Indeed, a degree of resistance to 
oncogene or by the TGF-α gene. which express higher levels of 

targeting drugs. we transduced and overexpressed the different 

expression of the RIα subunit of PKA and the sensitivity of 

formation of MCF-IOA cells with 

three Topo II inhibitory drugs (etoposide, teniposide, and am-

II-inhibited gene transfer.

The MCF-IOA RIα cells, but not the 

PKAI and cellular sensitivity to Topo II inhibitor drugs, but that 

this cannot be explained by a simple alteration in the expression 

activity of Topo II. This phenomenon does not appear to be 

restricted to the MCF-10A cells. In fact, we have recently 
demonstrated that ADR-5, a mutant of the Chinese hamster 

ovary cells, is hypersensitive to Topo II inhibitors and also 
expresses Rlα. Moreover, the transduction of Rlα cDNA into 

Chinese hamster ovary cells reconstitutes the hypersensitive 

phenotype of ADR-5 cells (35). It would be interesting to study 

whether other cell types overexpressing ras and/or TGF-α show 

hypersensitivity to Topo II inhibitors.

It has been shown recently that PKA may be implicated in 

the phosphorylation of human Topo IIα protein and that 

phosphorylation may alter enzymatic activity (36). Posttranslational 

modification or interference with the processing of Topo II-

catalyzed reactions may be involved in the hypersensitive 

phenotype of cells overexpressing the RIα subunit of PKAI. It is 

possible that there may be a convergence of signaling pathways 

involving PKA and Topo II, or alternatively that these proteins 

may affect common downstream targets since Topo II is able to 

interact directly with the cAMP-responsive element-binding 

protein and other related transcription factors (37). Another 

possible explanation is that the targeting of RIα overexpressing 
cells by Topo II inhibitors may depend on the evasion of a 

checkpoint since RIα favors cell entry into S phase. Although 

further work will be needed to determine if any of these sug-

gestions are true, our results are consistent with the RIα protein 

influencing drug sensitivity via modulation of events down-

stream of the Topo II-DNA cleavable complex. Our work may 

also provide a rationale for the study of new therapeutic modal-

ities combining conventional cytotoxic drugs with modulators 
of PKA activity. One such modulator, 8-CI-cAMP, a potent Rlα 
down-regulator, is currently under investigation in clinical trials 
in cancer patients.

REFERENCES


Cyclic AMP-dependent protein kinase type I is involved in hypersensitivity of human breast cells to topoisomerase II inhibitors.

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