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 amsacrine, 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 amsacrine 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, RIIβ or Ca, 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 constitutent 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 PKAII, termed R1α and R1β, and for two PKAI, termed RIIα and RIIβ, respectively (13). Among the different C isoforms, CB is the most widely studied in eukaryotic cells. Differential expression of PKAI regulatory subunits has been correlated with cell differentiation and neoplastic transformation (14). Preferential expression of RIIβ 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 accompanied 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 II α protein and are correspondingly more sensitive to Topo II-targeting drugs than are the parental cells (20).

In this study we have investigated 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 Rla 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 Rla 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 RIA, MCF-10A RIIβ, 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 RIA, RIIβ, and Ca genes, respectively. Expression of these genes was under the transcriptional control of the heavy metal-inducible mouse metallothionein-I promoter (22, 23). MCF-10A TGF-α, MCF-10A RIA, MCF-10A RIIβ, and MCF-10A Ca cells were continuously grown in the presence of 1 μM CdCl2 to maximally induce the expression of the metallothionein-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. Stock solutions (100X) of drugs were prepared immediately before use and diluted in medium. Treatments were carried out as described previously (24). After 6 days of culture, the cells were incubated at 37°C with MTT for 4 h, and readings of absorbance were carried out at 595 nm as described (24). For the clonogenic assay, 500 cells were plated in 60-mm dishes (Becton Dickinson Italia), treated with the appropriate drug concentrations, and colonies were counted after 7–10 days as described previously (25). To determine the IC50, linear regressions were plotted on the linear region of the curves. A mean value was calculated from a minimum of three experiments, each performed in six replicates, for each drug and cell line.

Western Blot Analysis. Protein lysates (50 μg total proteins/lane) were separated by SDS-PAGE on 4–20% gradient gels (Bio-Rad Laboratories, Milan, Italy), transferred to nitrocellulose, and the membranes were incubated either with a mouse monoclonal anti-human RIA antibody (kindly provided by Dr. B. Skhalegg, Rickshospitalet, Oslo, Norway), as previously described (26) or with T2K1, a polyclonal rabbit anti-serum raised against a synthetic 14-mer peptide representing human Topo IIa amino acid residues as described elsewhere (24).

RNase Protection Assay of Topo II. RNase protection assays were performed as described previously (11). The Topo IIα- and β-specific probes generated 215 and 228 baspairs protected fragments, respectively. An internal loading control of an antisense RNA to glyceraldehyde-3-phosphate dehydrogenase was used in each assay.

Determination of Topo II Levels by Flow Cytometry. Cells were trypsinized, washed twice with Ca2+/Mg2+-free PBS, and fixed in 100% methanol for 10 min at −20°C. Cells (105) were then incubated with 2 ml neutralizing buffer (Ca2+/Mg2+-free PBS + 2% goat serum) at room temperature for 15 min. After washing twice with Ca2+/Mg2+-free PBS, 50 μl anti-Topo II rabbit antiserum T2K1 (diluted 1:10 in Ca2+/Mg2+-free PBS plus 2% BSA) were added to each sample. Negative control samples contained non-immune rabbit serum instead of the anti-Topo II antibody. After 60 min at room temperature, the samples were washed twice with Ca2+/Mg2+-free PBS and 100 μl FITC-conjugated goat anti-rabbit antisera (Sigma) (diluted 1:30 in Ca2+/Mg2+-free PBS plus 2% BSA) were added to the samples. After 30 min at 4°C in the dark, cells were washed twice with Ca2+/Mg2+-free PBS and 1 ml propidium iodide staining solution (5 μl/ml propidium iodide in Ca2+/Mg2+-free PBS, pH 7.4) was added to the samples to perform the biparametric assay of DNA/Topo II content. Flow cytometric analysis was performed in duplicate with a FACSScan flow cytometer (Becton Dickinson, San Jose, CA) coupled with a Hewlett-Packard computer. A CONSORT 30 data analysis system (Becton Dickinson) was utilized for data acquisition.

Decatenation Assay of Plasmid DNA. Topo II catalytic activity was assayed in MCF-10A and MCF-10A RIA cells by measuring the decatenation of kinetoplast DNA from Crithidia fasciculata. Agarose gel electrophoresis and DNA visualization were carried out as described previously (25).

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).

To determine whether transformation by ras and TGF-α brought about overexpression of the RIA regulatory subunit of PKA, Western blotting analysis of cell extracts from MCF-10A, MCF-10A ras, and MCF-10A TGF-α cells was performed using an antihuman RIA monoclonal antibody. While in MCF-10A...
Fig. 1 Differential effect of etoposide, teniposide, and bleomycin and Western blotting analysis of Rλα expression on MCF-10A neo, MCF-10A ras, and MCF-10A TGF-α cells. 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. Western blot analysis was performed using a monoclonal antibody directed against the 49-kDa product of the Rλα subunit of PKA. Lane 1, MCF-10A neo cells; Lane 2, MCF-10A ras cells; Lane 3, MCF-10A TGF-α cells.

neo cells only a low level of the 49-kDa Rλα species was detected, MCF-10A ras and MCF-10A TGF-α cells showed a 20- and a 10-fold increase in Rλα protein levels, respectively (Fig. 1). No differences were observed in the growth pattern and the expression of Rλα protein in MCF-10A neo cells as compared to parental MCF-10A cells (data not shown).

To directly assess whether the overexpression of Rλα 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 Rλα, the RIIβ, or the Ca subunit of PKA (23). Western blotting analysis of cell extracts from MCF-10A neo, MCF-10A Rλα, MCF-10A RIIβ, and MCF-10A Ca cells using the antihuman Rλα monoclonal antibody showed that while in MCF-10 RIIβ and MCF-10A Ca cells the expression of Rλα protein was similar to that of MCF-10A neo cells, a 10- to 20-fold increase in the level of Rλα protein was observed in MCF-10A Rλα as compared to parental MCF-10A cells (Fig. 2).

The infected cells were then tested for sensitivity to cytotoxic drugs. MCF-10A Rλα cells exhibited hypersensitivity to etoposide and teniposide with approximately a 3-fold and 2-fold lower IC50 value, respectively, than MCF-10A neo, MCF-10A RIIβ, or MCF-10A Ca cells (Fig. 2). MCF-10 Rλα 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 Rλα protein (data not shown). MCF-10A RIIβ 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 Rλα cells as compared to MCF-10A neo, MCF-10A RIIβ, and MCF-10A Ca 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 Rλα 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 Rλα and MCF-10A ras cells (Fig. 3).
Hypersensitivity of Human Breast Cells to Topo II Inhibitors

Fig. 2  Differential effect of etoposide, teniposide, and bleomycin and Western blotting analysis of RIα expression in MCF-10A cells infected with different PKA subunits. MCF-10A neo, MCF-10A Ria, MCF-10A RIβ, and MCF-10A Ca 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 RIβ (Lane 2), MCF-10A Ca (Lane 3), and MCF-10A Ria (Lane 4) cells were tested with a monoclonal antibody directed against the 49-kDa product of the human RIα subunit of PKAI as described in “Materials and Methods.”

Fig. 3  Effect of etoposide and bleomycin on MCF-10A, MCF-10A Ria, 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 Rlx 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 Rlx 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 Rlx 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 Rlx 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 Rlx cells (Fig. 5).

**DISCUSSION**

Overexpression of the Rlx 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,
Hypersensitivity of Human Breast Cells to Topo II Inhibitors decreases with cell confluency (12, 33). MCF-10A 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 expression of the RIα subunit of PKA and the sensitivity of Topo lI-targeting drugs in human cells (28–32).

known that Topo II expression is cell cycle dependent and
calcinane). This result mimics the effect observed following trans-

teniposide, and amsacrine, but not to bleomycin, a drug which increased sensitivity of these transformed cells to Topo II activity or sensitivity to Topo II inhibitor drugs. The basis for this was the finding that mouse NIH 3T3 fibroblasts transformed by Ha-ras showed increased sensitivity to etoposide (20), and we had demonstrated previously that in two different rodent cell lines neoplastic transformation induced by ras or TGF-α is correlated with early induction of PKAI expression (18, 19).

In this study, we have demonstrated that human breast MCF-10A cells transformed by either a point mutated Ha-ras oncogene or by the TGF-α gene, which express higher levels of the RIα subunit of PKAI, became more sensitive to etoposide, teniposide, and amsacrine, but not to bleomycin, a drug which does not target Topo II. Indeed, a degree of resistance to bleomycin was observed in the transformed cells. To verify whether the overexpression of RIα could be involved in the increased sensitivity of these transformed cells to Topo II-targeting drugs, we transduced and overexpressed the different PKA subunits in MCF-10A cells by means of retroviral vector-mediated gene transfer. The MCF-10A RIα cells, but not the MCF-10AΔRIα or MCF-10AΔα cells, became hypersensitive to three Topo II inhibitory drugs (etoposide, teniposide, and amsacrine). This result mimics the effect observed following transformation of MCF-10A cells with ras and TGF-α genes. It is known that Topo II expression is cell cycle dependent and decreases with cell confluency (12, 33). MCF-10A cells over-

expressing c-Ha-ras, TGF-α, or RIα that grow in the presence of complete medium show the same cell cycle distribution and pattern of growth as parental MCF-10A (22, 23). Therefore, the increased sensitivity to Topo II drugs could not be explained on the basis of cell cycle distribution.

It has been shown in several human cell lines that levels of Topo II may account for differential sensitivity to Topo II inhibitory drugs and that low levels or mutated forms of Topo II may be responsible for acquired drug resistance (24, 25, 34). We therefore studied whether the levels and/or the activity of Topo II could be responsible for the hypersensitive phenotype in the different infectants of MCF-10A cells. However, we could not identify any difference in the levels of Topo II mRNA and protein between the drug-hypersensitive infectants and the parental MCF-10A cells. Moreover, analysis of Topo II catalytic activity by decatenation assay showed a similar level of Topo II enzyme activity in the nuclear extracts from MCF-10A and MCF-10 RIα cells.

Taken together, our results suggest that there is a direct relationship between the overexpression of RIα receptor of PKAI and cellular sensitivity to Topo II inhibitor drugs, but that this cannot be explained by a simple alteration in the expression or 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 overexpresses RIα. Moreover, the transduction of RIα cDNA into Chinese hamster ovary cells reconstitute 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 phospho-orylation 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 G1 checkpoint since RIα favors cell entry into S phase. Although further work will be needed to determine if any of these suggestions 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 modalities combining conventional cytotoxic drugs with modulators of PKA activity. One such modulator, 8-C1-cAMP, a potent RIα down-regulator, is currently under investigation in clinical trials in cancer patients.

REFERENCES


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