Induction of Topoisomerase II Gene Expression in Human Lymphocytes upon Phytohemagglutinin Stimulation

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Abstract

The carboxyl-terminal one-third of human topoisomerase II polypeptide expressed in Escherichia coli was used as antigen to generate polyclonal antibodies in rabbits. With the use of antiserum, DNA topoisomerase II levels of phytohemagglutinin-stimulated human lymphocytes were measured by immunoblotting. Our results showed that the increase in intracellular topoisomerase II level paralleled the entry of cells into proliferation. We also found that the increase in the topoisomerase II level resulted from an increase in the amount of topoisomerase II mRNA. The time course study indicated that the appearance of topoisomerase II mRNA was first observed at 36 h after phytohemagglutinin stimulation. The maximal level of topoisomerase II mRNA was seen at 45 h after stimulation. The same RNA blot was rehybridized with a thymidine kinase probe. The maximal level of thymidine kinase mRNA was observed at 39 h after phytohemagglutinin stimulation. In a comparison of the time course of topoisomerase II gene expression with that of thymidine kinase gene expression, it was found that the expression of the topoisomerase II gene was later than the onset of DNA replication. Thus, this study suggests that topoisomerase II, which is constantly expressed throughout the cell cycle, might participate in the initiation of DNA replication, while topoisomerase II is involved in solving the DNA topological problems accompanying DNA strand separation during DNA replication.

Introduction

The topological state of DNA is an important determinant for DNA structure and function (1–3). Two fundamentally different types of DNA topoisomerases have been found in human cells (4, 5). DNA topo I functions as a monomer to break and rejoin only one of the two DNA strands for each DNA strand-passing reaction, while DNA topo II functions as a homodimer to break and rejoin both strands for each DNA strand-passing reaction. Through these two fundamentally different mechanisms, the topological state of DNA can be modulated. Thus, both topo I and topo II are believed to play important roles in DNA metabolism, including transcription, replication, recombination, and chromosome segregation at mitosis (6–8). However, whether only one of the two or both topoisomerases are involved in the processes of transcription or replication is less clear at the present time. Previously, this laboratory found that the expression of the topo I gene, but not the topo II gene, could be induced in human skin fibroblasts upon phorbol ester stimulation (8). This observation may suggest that topo I, rather than topo II, is involved in solving the DNA topological problems generated during transcription. Recently, a possible link between topo II and cell proliferation has been reported (9–12). Topo II was thus implicated to have been involved in DNA replication. However, the expression of neither topo I nor topo II genes is exclusive when cells are in proliferation. In addition, both topo I and topo II are capable of relaxing supercoiled DNA. It becomes uncertain which one of the two or both topoisomerases are involved in solving the DNA topological problems accompanying strand separation during DNA replication. In this study, we have stimulated human lymphocytes with phytohemagglutinin for various lengths of time, followed by an examination of the accumulation of protein and mRNA levels of topo I and topo II. The results show that the topo I gene is constantly expressed throughout the cell cycle, while the expression of the topo II gene is later than the initiation of DNA replication. These observations may suggest that topo I participates in the initiation of DNA replication, while topo II is involved in solving the DNA topological problems accompanying DNA strand separation during DNA replication.

Materials and Methods

Materials. PHA, a mitogen for T-lymphocytes, was bought from Wellcome Diagnostics. DE-81, DEAE-cellulose ion-exchange chromatography paper, was obtained from Whatman. All tissue culture media were purchased from Gibco. Polyclonal antiserum against a recombinant human topoisomerase II polypeptide and human topo I and topo II cDNA clones were obtained as described previously (13, 14). 125I-Protein A and [methyl-3H]thymidine were purchased from NEN Research. Reagents for gel electrophoresis were from Bio-Rad or Bethesda Research Laboratories. All other chemicals were analytical reagent grade.

Cell Culture. Blood obtained from a healthy donor was mixed with heparin sulfate (20 units/ml). Human lymphocytes were isolated from heparanized blood by Ficoll-Hypaque density gradient centrifugation (15). Human lymphocytes were then cultured at a density of 2 × 10⁶ cells/ml in suspension using RPMI 1640 containing 10% fetal bovine serum, penicillin/streptomycin (50 IU/ml and 50 μg/ml, respectively), and 1 mM glutamine in a controlled atmosphere of 5% CO₂ and 95% air (v/v) at 37°C. For PHA stimulation, PHA was added at the concentration of 0.1 μg/ml in the beginning of cell culture.

Thymidine Incorporation. Human lymphocytes isolated from peripheral blood of healthy donors were treated with PHA. At the various times of PHA stimulation, 1 μCi of [methyl-3H]thymidine was added to aliquots of lymphocytes (10⁶ cells each). Two h later, the cells were pelleted and washed with PBS. Cell pellets were lysed with 100 μl of 0.2% SDS and spotted onto DE-81 filter paper (2 x 2 cm). DE-81 papers were then washed with 0.3 M ammonium acetate 3 times, ethanol once, and ethyl ether once. The filters were dried in air, and the radioactivity was determined by scintillation counting.

Immunoblotting. Human lymphocytes were stimulated with PHA. Aliquots of cells (10⁶ cells) were withdrawn at various times and washed with PBS. Cell pellets were dissolved in Laemmli buffer and boiled for 5 min prior to application to a 0.1% SDS:7.5% acrylamide slab gel as described by Laemmli (16). For immunoblotting, electrophoresed samples were transferred from gel to nitrocellulose membrane at 10 V/cm for 8 h at 4°C in Tris-glycine buffer (26 mM Tris; 192 mM glycine, pH 8.2) containing 0.2% SDS and 20% methanol. After transfer, blots were soaked in PTX buffer containing 1% gelatin for 1 h and then incubated with anti-topo II antiserum (1:500 dilution) in PTX overnight.
at room temperature. Following incubation, blots were washed 4 times in PTX, once in urea wash (2 M urea:1% Triton X-100:100 mM glycine), and 2 additional times in PTX. Blots were then incubated with 125I-Protein A (5 x 10^3 cpm/ml in PTX) for 1 h and washed as before. The blots were dried in air and visualized by autoradiography.

RNA Isolation and RNA Blotting. At various times, human lymphocytes were removed from cultures, and total cytoplasmic RNA was isolated according to the method described by Wilkinson (17). RNA was quantified by the measure of absorbance at 260 nm. Samples (15 μg of RNA/well) were fractionated on 1% agarose/6% formaldehyde gels. Before RNA was transferred to nitrocellulose membranes, gels were stained with ethidium bromide to assess the integrity of RNA and also to verify that equal amounts of RNA were loaded for each sample. After RNA was transferred to the nitrocellulose membrane, the membrane was visualized under UV light to check the uniformity of the RNA transfer and to locate the 28S and 18S rRNA bands. Filters were dried, baked at 80°C for 3 h, prehybridized at 42°C for 4 h, and hybridized overnight with a nick-translated 32P-labeled cDNA of human thymidine kinase, topo I, and topo II (18). The specific activity of the probe was approximately 10^8 dpm/μg of DNA. After washing twice with 2 x SSC plus 0.1% SDS at 50°C and once with 0.1 x SSC plus 0.1% SDS at 55°C, the filter was dried in air and exposed to Kodak XAR film at ~70°C with an intensifying screen.

Results and Discussion

There is now substantial evidence indicating that one or more DNA topoisomerases are involved in DNA replication (19-21). In *E. coli* as well as in yeast, it is well established that topo II is essential, while topo I is not. These observations may imply that topo II alone is sufficient in solving the topological problems accompanying DNA strand separation during DNA replication. Furthermore, the results of *in vitro* studies also indicate that topo II alone is sufficient in solving the DNA topological problems during replication (22-24). Thus, it is unclear whether topo I normally participates in DNA replication. To examine the involvement of topo I in DNA replication, we studied the kinetics of topo II gene expression and its time course in relation to the initiation of DNA replication. If the expression of the topo II gene was later than the onset of DNA replication, topo I might then be involved in solving DNA topological problems during the initiation of DNA replication. We had chosen human lymphocytes for these studies. The advantage of using human lymphocytes was that, when isolated from blood, lymphocytes were in the G0 stage of the cell cycle. Upon PHA stimulation, resting (G0) cells were induced to enter the cell cycle where the lymphocytes became a defined population and synchronously underwent a programmed developmental change. Thus, when cells began to incorporate thymidine into DNA, it was thought to be the time of initiation of DNA replication. In order to determine the onset of DNA replication, we exposed human lymphocytes to phytohemagglutinin for various lengths of time, followed by an examination of the [3H]thymidine incorporation. As shown in Fig. 1, [3H]thymidine incorporation was undetectable up to 18 h of PHA stimulation. An observable increase of [3H]thymidine incorporation was detected at 24 h and reached its maximum after 36 h of PHA stimulation. This increase was followed by a rapid fall, but not down to the basal level even after 54 h of PHA stimulation. These results indicated that DNA replication was initiated at 24 h for certain populations of the PHA-stimulated T-lymphocytes and at 30 h for the majority of the PHA-stimulated T-lymphocytes.

The cellular level of topo II in human lymphocytes after PHA stimulation was measured by immunoblotting using rabbit antiserum against the recombinant human top II polypeptide (13).

Prior to PHA stimulation, the level of topo II was undetectable in human lymphocytes even after overexposure of the autoradiograph (Fig. 2). Upon PHA stimulation, the level of topo II started to increase gradually. A detectable level of topo II was first observed at 34 h of PHA stimulation. Between 42 and 48 h after PHA stimulation, the level of topo II reached its maximum. We then examined whether the increase in topo II resulted from an increase in the amount of topo II mRNA. RNA was isolated from human lymphocytes with pretreatment of PHA for 36, 39, 42, 45, and 48 h. Their content of topo II mRNA was then determined by hybridizing the Northern blot to topo II cDNA probe (13). As shown in Fig. 3A, the RNA gel after ethidium bromide staining exhibits equal intensity of both 28S and 18S rRNA bands for all RNA samples, indicating that an equal amount of RNA was loaded for each sample. RNA was transferred to nitrocellulose membrane and hybridized to topo I and topo II cDNA probes (Fig. 3B). The level of topo II mRNA remained fairly constant throughout the time period of study, while the level of topo II mRNA increased during the course of PHA stimulation. A very faint topo II mRNA band was observed in lymphocytes at 36 h after PHA stimulation. With longer PHA stimulation, the level of topo II mRNA increased further. A 10- to 15-fold increase in topo II mRNA was observed 39 h after PHA stimulation in comparison with the level 36 h after PHA stimulation. The increase in the topo II mRNA level reached its maximum at 45 h of PHA treatment. These results demonstrated that the increase in topo II resulted from an increase in topo II mRNA.

The kinetic studies of topo II gene expression using both immunoblotting and RNA analysis indicated that the expression of the topo II gene was somewhat later than the onset of DNA replication. To further confirm this observation, the same Northern blot was rehybridized to a thymidine kinase probe. The maximal level of thymidine kinase was observed in human lymphocytes at 39 h after PHA stimulation (Fig. 3B). Since the expression of the thymidine kinase gene was at the G1-S boundary (25), comparing the time course of topo II gene expression with that of thymidine kinase gene expression again indicated that the expression of the topo II gene was later than the onset of DNA replication. Thus, the results of these kinetic studies of topo II gene expression may suggest that topo I participates in the initiation of DNA replication, while topo II is involved in solving the DNA topological problems accompanying DNA strand separation during DNA replication.

It may be argued that a few molecules of topo II are sufficient in solving the DNA topological problems generated during the
Induction of Topoisomerase II Expression by PHA

Fig. 2. Induction of DNA topoisomerase II in PHA-stimulated human lymphocytes. Aliquots (10⁶ cells) of PHA-stimulated lymphocytes were prepared for immunoblotting. Samples were electrophoresed and immunoblotted with antiserum against recombinant human top II carboxyl-terminal polypeptide. kD, kilodalton.

Fig. 3. Quantitative analysis of topo I mRNA in PHA-stimulated human lymphocytes. A, RNA gel after ethidium bromide staining exhibits 28S and 18S rRNA bands. B, human lymphocytes were stimulated with PHA for the indicated times (36 to 48 h). The levels of topo I, topo II, and thymidine kinase mRNAs in response to PHA stimulation were then analyzed by RNA blot hybridization to labeled human topo I, topo II, and thymidine kinase cDNA probes.

initiation of DNA replication. Therefore, cells only need to synthesize a few topo II molecules at the beginning of DNA replication. Since the sensitivity of immunoblotting and Northern blotting has its limits, we may not be able to detect the expression of the topo II gene at the beginning of DNA replication. However, it may still be possible that late topo II gene expression is indeed due to the fact that only topo I is needed at the beginning of DNA replication. This possibility is consistent with the finding reported by Kim and Wang (26), that the absence of topo I leads to a temporary delay in the extension of the short DNA chain and that this delay in chain elongation is also reflected in the rate of total DNA synthesis in the topo I gene deletion mutant during the early S phase in yeast. Thus these observations further support our belief that topo I participates in the initiation of DNA replication, while topo II is involved in solving the DNA topological problems accompanying DNA strand separation during DNA replication.

Acknowledgments

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References

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*Cancer Res* 1990;50:5649s-5652s.