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Immunohistochemical Study of DNA Topoisomerase II in Human Gastric Disorders

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Topoisomerase II (topo II) separates chromosomes at the end of mitosis and is also the target for various chemotherapeutic agents. Expression of this enzyme has been demonstrated to increase rapidly at the end of the S to G2/M phase and decrease after the completion of mitosis. We immunolocalized topo II in specimens of both normal and neoplastic human gastric mucosas to evaluate expression of this enzyme. Three different antibodies were used for the immunostaining of topo II (anti-topo IIα isoform, anti-topo IIβ isoform and anti-topo IIα and -β isoforms). There were no significant differences in topo II labeling index (LI) between frozen and paraffin-embedded tissue sections obtained from the same cases. Topo II LI was significantly correlated with Ki67 LI in all of the specimens examined. The area of cells positive for Topo II was much narrower than that of Ki67 in the normal gastric glands, and the pattern of Topo II immunolocalization in both adenomas and adenocarcinomas was also essentially the same as that of Ki67. The topo II LI values (positive cells/1000 cells) for normal gastric gland, adenoma, intestinal-type adenocarcinoma, and diffuse-type adenocarcinoma were 114.7 ± 2.2, 266.7 ± 18.8, 277.6 ± 19.2, and 324.5 ± 5.3, respectively. Significant differences in topo II LI and topo II/Ki67 index were observed between normal and neoplastic mucosas ($P < 0.0001$) and between adenomas or intestinal-type adenocarcinoma and diffuse-type adenocarcinoma ($P < 0.001$ and $P < 0.01$, respectively). Simultaneous measurement of topo IIα and nuclear DNA content by two-parameter flow cytometry revealed that the Jurkat cell line established from acute lymphocytic leukemia cells expressed the enzyme in cells at other than S and G2/M phases of the cell cycle whereas topo-IIα-positive cells were predominantly observed in S and G2/M phases of the cell cycle in the cells from normal lymph nodes. These findings suggest that dysregulation or qualitative changes of topo IIα expression are associated with malignancy. Topo II immunostaining can thus detect proliferating cells in routinely processed tissue sections and can indicate the altered topo IIα expression in human cancers, which may be related to the sensitivity to topo-II-targeted chemotherapeutic agents. (Am J Pathol 1996, 149:997–1007)

The analysis of cell proliferation *in situ* is important to the anatomic pathologist in evaluating cell turnover in various tissues and organs. Methods for studying cell proliferation *in situ* include the mitotic count,[1] [^{3}H]thymidine or 5-bromo-2′-deoxyuridine (BrdU) incorporation,[2] analysis of S-phase fraction by image cytometry,[3–5] and immunohistochemistry of cell-cycle-related nuclear proteins.[6,7] These methods have advantages and disadvantages in their application to surgical pathology materials. The immunohistochemistry of cell-cycle-related nuclear proteins, especially that of a nuclear antigen recognized by a mouse monoclonal antibody Ki67, is widely used in histopathology laboratories to assess cell proliferation.[6,8] This method is straightforward, is relatively rapid and easy, and can be applied to routinely processed diagnostic surgical pathology materials when employing the monoclonal antibody MIB-1[9] and microwave irradiation for antigen retrieval.[10]

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Ki67 positivity in tissue sections may therefore provide an indication of the number of cells cycling at any time, ie, cells at other than G) and early G1.[11,12] However, Ki67 immunostaining cannot determine at which stage of the cell cycle these positive cells are.

DNA topoisomerase II (topo II) separates chromosomes at the end of mitosis.[13] Topo II causes a transient break of double-stranded DNA through which another DNA molecule is passed. After passage of the strand has been completed, the enzyme relinks the transient DNA. *In vivo* studies have demonstrated that the concentration of topo II increases rapidly at the end of the S to G2/M phase and decreases rapidly when mitosis has been completed of non-neoplastic proliferative cells.[14–17] Therefore, the presence or expression of topo II may indicate that the topo-II-positive cells are in the S to M phase of the cell cycle of these cells. Little information exists on topo II expression or activity in human neoplasms, especially in human solid tumors. However, topo II is also well known as a target for various chemotherapeutic agents,[18] such as etoposide, teniposide, 4′-(9-acridinylamino)methanesulfon-m-anisidide (m-AMSA), doxorubicin, ellipticine, and mitoxantrone.[19–21] Both *in vivo* and *in vitro* data demonstrate that cellular resistance or sensitivity to the topo II poisons depends on the nuclear level of topo II in various cell lines established from human cancers.[22–26] Analysis of the nuclear levels of topo II in tissue specimens from human cancers may therefore provide important information about the potential efficacy of chemotherapeutic agents. In this study, we performed immunostaining for topo II in surgical pathology specimens of the human stomach, including normal tissue, adenoma, and carcinoma, using three different monoclonal antibodies against topo II. We calculated the labeling index (LI) of topo II in these lesions by counting 1000 cells. Immunohistochemical findings were then correlated with the results of Ki67 immunostaining in serial sections to evaluate whether topo II can serve as an immunohistochemical marker of proliferating cells and to determine the possible correlation of increased topo II immunoreactivity with malignant phenotype of human stomach. In addition, we examined simultaneous measurement of topo IIα and nuclear DNA content by two-parameter flow cytometry in the Jurkat cell line established from human acute lymphocytic leukemia cells[38] and nonmetastatic lymph nodes to examine any possible qualitative differences of topo IIα expression between neoplastic and non-neoplastic cells.

Materials and Methods

Specimens of Human Stomach

A total of 81 specimens of the stomach obtained from Japanese patients were evaluated. Specimens included normal gastric mucosa (17 cases), gastric adenoma (17 cases), and adenocarcinoma (48 cases). Specimens were obtained from the patients in Tohoku University Hospital and its affiliated hospitals in Sendai, Japan. Nonpathological gastric mucosa was taken from non-atrophic areas of surgical specimens of the human stomach with adenocarcinoma. Specimens of adenomas and carcinomas were obtained at endoscopic mucosal resection (adenoma, 17 cases; carcinoma, 13 cases) or gastrectomy (carcinoma, 35 cases). Adenocarcinomas were classified histopathologically according to the criteria of Lauren[27] as follows: intestinal type (32) and diffuse type (16). The normal mucosa was selected from histologically defined non-atrophic, nonmetaplastic, and non-inflammatory gastric mucosas.

Processing of Tissue

Tissue blocks were trimmed to 1.5 × 1.5 × 0.3 cm and immediately fixed in 8% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, or in 10% formalin for 18 hours at 4°C. They were then frozen or embedded in paraffin. Frozen tissue sections were prepared from 17 cases of the nonpathological gastric mucosa. A portion of unfixed tissue or of tissue fixed in 8% paraformaldehyde was embedded in OCT compound (Miles, Elkhart, IN) after the specimen had been placed sequentially in 10, 15, and 20% sucrose in PBS for 6 hours each at 4°C. Specimens obtained from endoscopic mucosal resection were examined carefully and sectioned to 0.5 cm wide under a microscope and fixed in 10% formalin for 18 hours at room temperature. They were then embedded in paraffin.

Preparation of Monoclonal Antibodies

Details of cloning and subsequent production of antibodies against topo II are reported by the group of Kikuchi et al (manuscript in preparation). Briefly, the cDNA of human topo IIα was expressed in *Escherichia coli* under the control of the T7 promoter, and the protein produced was injected four times every 2 weeks into a Balb/C mouse. The splenocytes were fused with the myeloma cell line P3-X63Ag8, 653, and the culture media were screened by enzyme-linked immunosolvent assay. Clone 7B9 was IgG2b

subtype and recognized both topo IIα and topo IIβ. Its epitope was located in the region of 111 to 244 amino acid residues from the amino terminus of human topo IIα. Monoclonal antibody 8D2 was prepared as described above, except that a partially purified preparation of human topo II (a mixture of IIα and IIβ) was used. This clone was IgG2b subtype and recognized exclusively human topo IIα (but not IIβ); its epitope was in the region of 1258 to 1448 amino acid residues. Clone 5A7 was specific against a synthetic peptide found near the carboxyl-terminal region of human topo IIβ and its subtype was IgG2b. The antibody was specific to the β-isoform of human topo II.[28]

Immunoblotting

A nuclear extract of HL60 cells was lysed in sodium dodecyl sulfate (SDS)-PAGE sample buffer by boiling it for 90 seconds. Lysates were subsequently separated on 7.5% polyacrylamide gel. In each lane, a nuclear extract containing approximately 50 μg of protein was used for analysis. After electrophoresis, protein bands were transferred by use of a semi-dry blotting apparatus to polyvinyl difluoride membrane (Millipore Japan, Tokyo, Japan) in a buffer containing 48 mmol/L Tris base, 39 mmol/L glycine, 0.037% SDS, and 20% methanol at 150 mA for 60 minutes. The membrane was first blocked with 10% skim milk suspended in Tris buffer solution (TBS), containing 10 mmol/L Tris-HCl, pH 8.0, 0.15 mmol/L NaCl, for 2 hours at 37°C, washed briefly with TBS, and incubated with the primary antibody (1:10 dilution) in TBS for 15 hours at 4°C, with gentle shaking. The filter was rinsed extensively with TBS containing 0.1% Tween (TBST) and then incubated with the secondary antibody, horseradish-peroxidase-conjugated anti-mouse IgG (Bio-Rad, Richmond, CA) at a 3000-fold dilution for 2 hours at 37°C. The filter was washed with three changes of TBST and two changes of TBS. Signals were detected by use of a Konica immunostaining kit (Konica Co., Tokyo, Japan) with 2-chloronaphthol.

Immunohistochemistry

Thin (2.5-μm) slices from paraffin-embedded specimens were deparaffinized routinely. Ki67 and topo II immunostaining was performed in serial sections of the same blocks. Frozen sections (5 μm thick) were prepared on a cryostat and air dried for 30 minutes at room temperature. Frozen sections from nonfixed specimens were fixed in acetone for 10 minutes at 4°C after air drying. Paraffin-embedded slides were then autoclaved for 5 minutes in 10 mmol/L sodium citrate buffer (pH 6.0). After the slides had been kept for 1 hour at room temperature, they were immersed in ethanol with 0.3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. In frozen sections, endogenous peroxidase activity was blocked for 15 minutes before incubation with the secondary antibody, as was reported previously.[29] Sections were washed three times with PBS, 0.01 mol/L, for 5 minutes each time and treated with 1% normal rabbit serum for 30 minutes at room temperature. After being washed, the slides were incubated with the primary antibodies for 18 hours at 4°C in a humidified chamber. Monoclonal antibody Ki67 (MIB-1) was purchased from Immunotech (Hamburg, Germany). Optimal dilution for the antibodies was 1:300 for anti-topo II antibodies and 1:50 for Ki67, as determined by the preliminary studies. Histofine Kit (Nichirei Co., Tokyo, Japan) was used in the immunostaining. Histofine Kit was composed of biotinylated rabbit anti-mouse immunoglobulin and peroxidase-conjugated streptavidin. After washing, the slides were incubated for 30 minutes at room temperature with biotinylated rabbit anti-mouse immunoglobulin and peroxidase-conjugated streptavidin. Between incubations, slides were washed with PBS, 0.01 mol/L. A final wash was followed by their immersion for 5 to 10 minutes in a solution containing 3-3′-diaminobenzidine, 0.06 mmol/L, and hydrogen peroxide, 2 mmol/L, in Tris-HCl, 0.05 mol/L, buffered at pH 7.6. Sections were counterstained with 1% methyl green and mounted in a glycerol gelatin water-soluble medium. For the negative control of the immunostaining, PBS, 0.01 mol/L, or non-immunized mouse IgG was used instead of primary antibodies.

Antigen Retrieval in Paraffin-Embedded Tissue Sections

In this study, in addition to autoclave treatment described above, we applied the following methods to paraffin-embedded tissue sections for antigen retrieval: digestion with proteinase (0.5 mg/ml for 20 minutes at 37°C; Sigma Chemical Co., St. Louis, MO), trypsin (1 mg/ml for 30 minutes at 37°C; Wako Pure Chemical Industries, Tokyo, Japan), pepsin (0.2 mg/ml for 30 minutes at 37°C; Sigma), or a mixture of the above after deparaffinization; microwave irradiation for 15 minutes (500 W Hitachi MR-M26) in 10 mmol/L sodium citrate (Sigma), pH 6.0; and digestion with proteinase (0.5 mg/ml for 20 minutes at 37°C; Sigma) after microwave irradiation or heating in an autoclave for 10 minutes at 121°C.

Table 1. *Clinical and Histopathological Data, Ki67 and Topo II LI and Topo II/Ki67 Index of Normal Gastric Mucosa, Adenoma, and Carcinoma*

	Age (median years)	Sex (M/F)	Ki67 LI (mean ± SE)	Topo II LI (mean ± SE)	Topo II/Ki67 index (mean ± SE)
Normal mucosa (n = 17)	66.9 ± 10.3	13/4	269.6 ± 2.5	114.7 ± 2.2	42.6 ± 0.48
Adenoma (n = 17)	66.0 ± 8.8	14/3	486.1 ± 27.9	266.7 ± 18.8	54.7 ± 1.9
Carcinoma (n = 48)					
Intestinal (n = 32)	62.13 ± 8.6	26/6	496.6 ± 16.8	277.6 ± 10.2	55.8 ± 3.1
Non-intestinal (n = 16)	62.1 ± 11.3	9/7	501.7 ± 9.9	324.5 ± 5.3	64.8 ± 0.52

Ki67 and Topo II LI represent the number of positive cells per 1000 cells examined. PPR index represents Topo II LI/Ki67 LI (%). M, male; F, female.

Two-Parameter Flow Cytometry

Simultaneous measurement of topo IIα and nuclear DNA content was done by two-parameter flow cytometry, according to the method of Goukon et al[30] with some modifications. The Jurkat cell line established from acute lymphocytic leukemia cells[31] and nonmetastatic lymph nodes from the surgical pathology specimens submitted to the Department of Pathology, Tohoku University Hospital, were minced in PBS containing 0.1% Triton X-100 (Sigma) at 4°C. The suspension was washed in 0.01 mol/L PBS three times and centrifuged at 1500 rpm at 4°C. The pellet was fixed in 70% ethanol for 15 minutes at 4°C. After washing three times in 0.01 mol/L PBS and centrifugation at 1500 rpm, the pellet was resuspended in 2% non-immunized goat serum (Nichirei) in 0.01 mol/L PBS for 45 minutes at room temperature. The pellet was aliquoted into two plastic tubes. In one tube, the sample was incubated with monoclonal anti-topo IIα antibody at a 1:100 dilution for 18 hours at 4°C. In the other tube, the sample was incubated with 0.01 mol/L PBS containing 0.5% bovine serum or non-immunized mouse IgG for 18 hours at 4°C instead of the primary antibody as a negative control. Fluorescein-5 isothiocyanate (FITC)-conjugated $F(ab')_2$ fragment of goat anti-mouse immunoglobulins (Dako, Glostrup, Denmark) was used as the secondary antibody. The samples were incubated with the secondary antibody for 45 minutes at room temperature in the dark. They were then washed extensively in 0.01 mol/L PBS and centrifuged at 1500 rpm. The samples were then incubated in 1 mg/ml RNAse (Sigma) for 30 minutes and stained with 10 μg/ml propidium iodide (PI; Sigma) for 30 minutes at 4°C for DNA staining. As for the control of DNA content, the sample was treated with RNAse and stained by PI after fixation. The samples were subsequently analyzed on a Mac-FACScan flow cytometer (Becton Dickinson, Mountain View, CA) after filtration through a 50-μm nylon mesh. Excitation was achieved with a 488-nm argon laser, green fluorescence (FITC) was measured at 510 to 550 nm, and red fluorescence (PI) was measured at 580 to 620 nm.

Counting and Statistical Analysis

There was some variation in the intensity of the nuclear immunostain in different positive cells. Therefore, all positively stained nuclei, regardless of staining intensity, were scored as positive. Scoring of topo II in tissue sections incubated with the anti-topo IIα subunit was performed in a high-power field (×400) by two of the authors (N. Yabuki and H. Sasano), independently. In each case, 1000 cells were counted in the lesions. Inter- and intraobserver differences were less than 5%. Discordant cases were re-evaluated together by two of the authors above with a double-headed microscope (BH2, Olympus Co., Tokyo, Japan). We also determined the topo II/Ki67 index, ie, the topo IIα index divided by the Ki67 index in the same lesions of specimens.

Values were presented as mean ± SE. The significance of differences was tested by the Mann-Whitney test. The criterion for statistical significance was $P < 0.05$.

Results

Immunostaining

Results are summarized in Table 1. In both specimens of frozen and paraffin-embedded sections, immunoreactivity for Ki67 and topo II was observed in the nuclei (Figures 1 to 4).

Correlation among Different Antibodies

Only the anti-topo IIα and anti-topo IIα and -β antibodies showed nuclear immunoreactivity in all tissue preparations; the anti-topo IIβ antibody did not demonstrate any immunoreactivity. Among the different methods used for antigen retrieval in paraffin-embedded tissue sections, only the autoclaving method

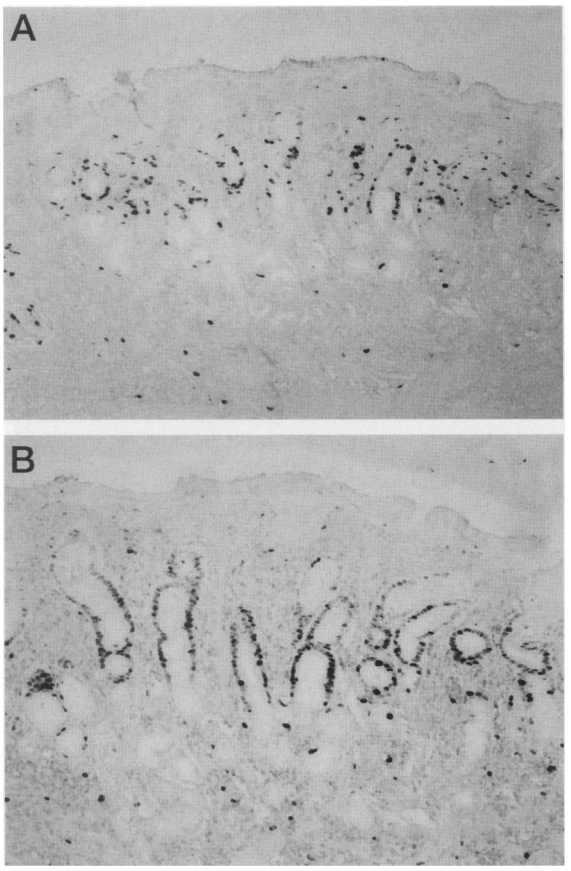

Figure 1. *Immunohistochemistry of topo II using anti-α-subunit monoclonal antibody* (**A**) *and Ki67* (**B**) *in 10% formalin-fixed and paraffin-embedded tissue sections of normal gastric mucosa* (*68-year-old male*). *Both topo II and Ki67 immunoreactivities were located in the nuclei of the cells at the neck of foveolar glands, but the cells positive for Ki67 were much more widely distributed than those positive for topo II. Magnification, ×200.*

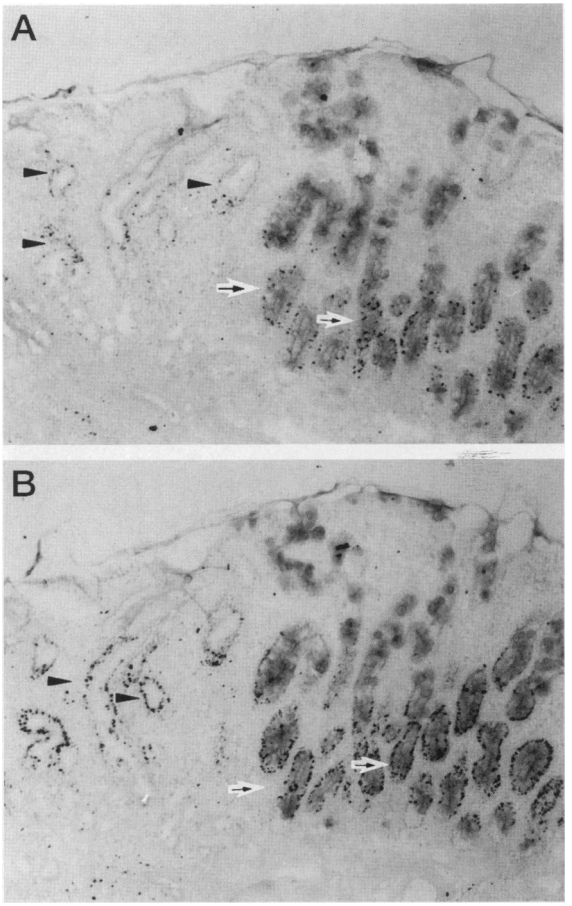

Figure 2. *Immunohistochemistry of topo II using anti-α-subunit monoclonal antibody* (**A**) *and Ki67* (**B**) *in unfixed and frozen tissue sections of non-neoplastic gastric mucosa* (*68-year-old male*). *Alcian blue staining of PH2.5 was performed after immunostaining. Both topo II- and Ki67-positive cells were predominantly distributed in the neck of foveolar glands* (**arrowheads**) *of nonmetaplastic mucosa and in the bottom of gastric glands with intestinal metaplasia in which alcian-blue-positive cells were present* (**arrows**). *As in Figure 1, cells positive for Ki67 were much more widely distributed than those positive for topo II. Magnification, ×200.*

and digestion with trypsin after microwave irradiation resulted in nuclear immunoreactivity of topo II. However, the autoclave method yielded fewer background signals than did the method of microwave irradiation.

Correlation among Different Tissue Preparations

In normal or non-neoplastic human stomach, cells positive for topo II were present mainly in the neck of the foveolar epithelium compartment and in the proliferative zone in both the frozen and paraffin-embedded tissue sections (Figures 1A and 2A). There was a significant correlation of topo II LI between the frozen and paraffin-embedded tissue sections in the 17 cases of non-neoplastic gastric mucosa (Figure 5). Topo II immunoreactivity was consistently observed in the germinal center of the gastric mucosa.

Correlation with Ki67 Nuclear Antigen Immunostaining

There was a significant positive correlation between the Ki67 LI and topo II LI in normal gastric mucosa, adenoma, and carcinoma ($P < 0.001$; Figure 6). Ki67 LI values were significantly higher than those of topo II LI when evaluating all normal mucosa, adenoma, and carcinoma cases. In the normal or non-neoplastic gastric mucosa, the patterns of Ki67 immunolocalization were essentially the same as those of topo II as demonstrated in Figures 1 and 2. However, the area of cells positive for Ki67 was much wider than that of topo II in the gastric glands (Figures 1 and 2). Patterns of Ki67 immunolocalization in adenomas and carcinomas were also essentially the same as those of topo II as demonstrated in Figures 3 and 4.

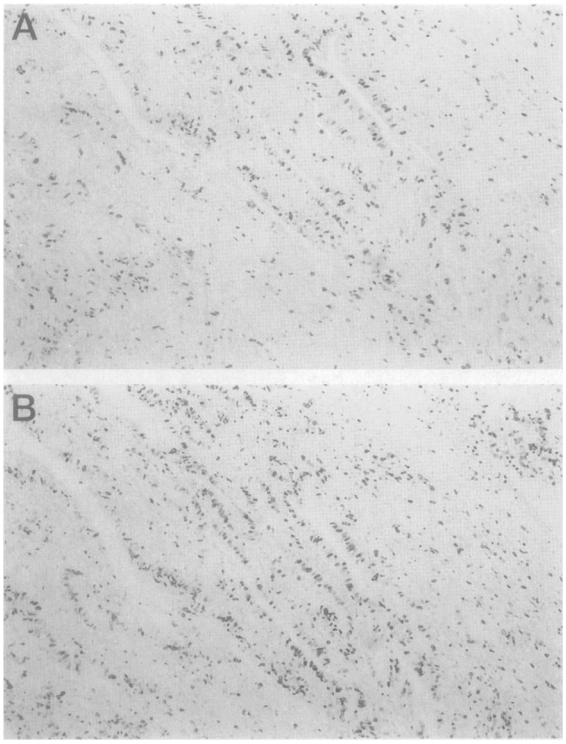

Figure 3. *Immunohistochemistry of topo II using anti-α-subunit monoclonal antibody* (A) *and Ki67* (B) *in 10% formalin-fixed and paraffin-embedded tissue sections of gastric adenoma (75-year-old male). Magnification, ×200.*

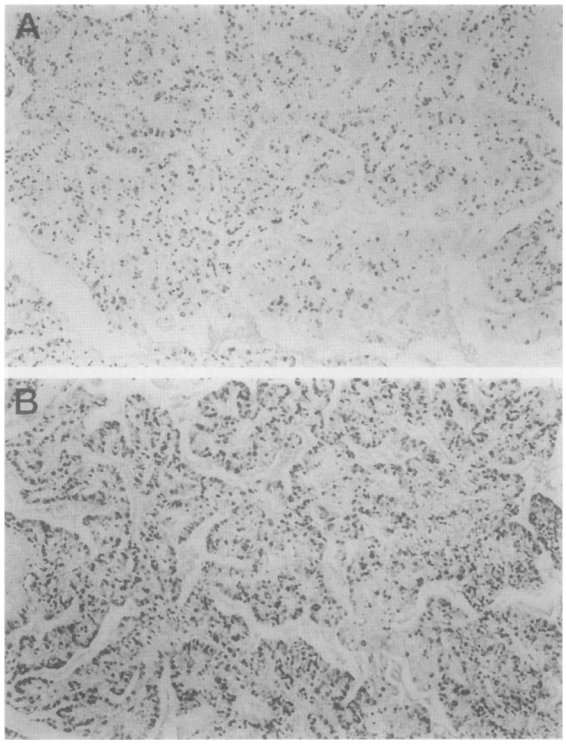

Figure 4. *Immunohistochemistry of topo II using anti-α-subunit monoclonal antibody* (A) *and Ki67* (B) *in 10% formalin-fixed and paraffin-embedded tissue sections of intestinal-type gastric adenocarcinoma. (65-year-old male.) Magnification, ×200.*

Examination of serial tissue sections revealed that topo-II-positive cells were nearly always positive for Ki67 but not vice versa in all of the specimens examined including normal stomach, adenoma, and carcinoma. Control tissue sections incubated with PBS, 0.01 mol/L, or normal mouse IgG did not yield immunoreactivity.

Immunoblotting

Results of immunoblotting are summarized in Figure 7. The 7B9 antibody recognized two different bands in immunoblotting, 170 and 180 kd, corresponding to the molecular weights of topo IIα and -β, respectively. The monoclonal antibody 8D2 recognized the 170-kd band of topo IIα and the monoclonal antibody 5A7 recognized the 180-kd band of topo IIβ.

Two-Parameter Flow Cytometric Analysis

Single-parameter histograms and two-parameter dot plots were demonstrated in Figure 8. Topo-IIα-positive cells were defined as those expressing higher FITC levels than that in the negative control samples (Figures 8, A and B). A positive correlation was observed between topo IIα expression and DNA content in both Jurkat cells and lymphocytes obtained from lymph nodes. In Jurkat cells, positive cells were detected in both DNA aneuploid and diploid cell populations in a two-parameter dot plot (Figure 8C). In Jurkat cells, topo-IIα-positive cells were observed in cells at other than S and G2/M phases of the cell cycle (Figure 8C). In nonmetastatic lymph nodes, the topo IIα expression level was not greater than the background levels in the great majority of the cells examined, possibly due to the relatively small num-

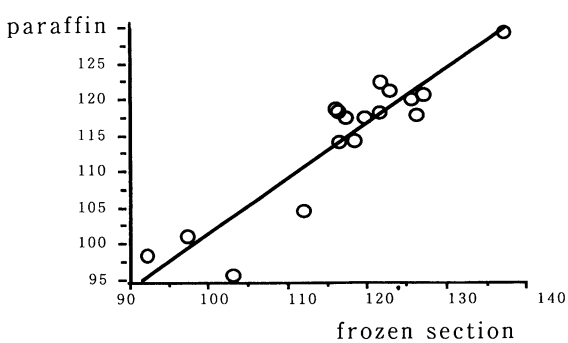

Figure 5. *Correlation of topo II LI between 8% paraformaldehyde-fixed, paraffin-embedded and frozen tissue sections of non-neoplastic gastric mucosa. The* x *and* y *axes represent topo II LI, ie, the number of positive cells per 1000 cells. There was a significantly positive correlation observed* ($y = 25.08 + 0.7765x$; $r^2 = 0.849$).

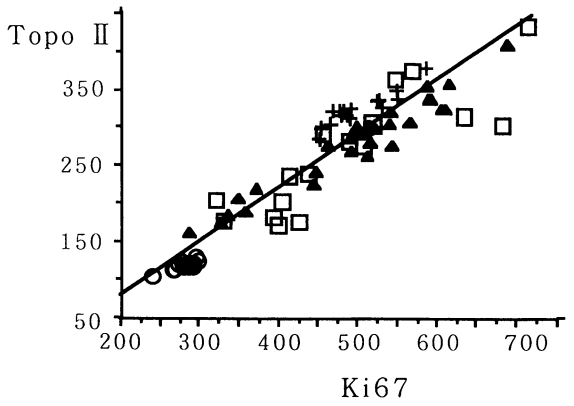

Figure 6. *Correlation between Ki67 and topo II LI in 10% formalin-fixed and paraffin-embedded tissue sections of normal mucosa (○), adenoma (□), intestinal-type adenocarcinoma (△), and diffuse-type adenocarcinoma (+). The x and y axes represent Ki67 and topo II LIs, ie, the number of positive cells per 1000 cells examined, respectively.*

ber of proliferative cells in the lymph nodes (Figure 8D). However, no distinctive groups of topo-IIα-positive cells were detected in G1 in a two-parameter dot plot (Figure 8D).

Topo II LI among Normal Epithelium, Adenoma, and Carcinoma

There were significant differences in topo II LI between the neoplasms (including adenoma and adenocarcinoma) and the normal mucosa ($P < 0.001$) and between adenoma or the intestinal and the diffuse types of adenocarcinoma ($P < 0.01$ and $P < 0.001$, respectively; Table 1). Diffuse-type gastric adenocarcinoma demonstrated a significantly higher topo II LI than either the adenomas or intestinal-type adenocarcinomas. Significant differences in the topo II/Ki67 index were observed between the normal mucosa and neoplasm ($P < 0.001$) and between adenoma or intestinal-type carcinoma and diffuse-type carcinoma ($P < 0.0001$).

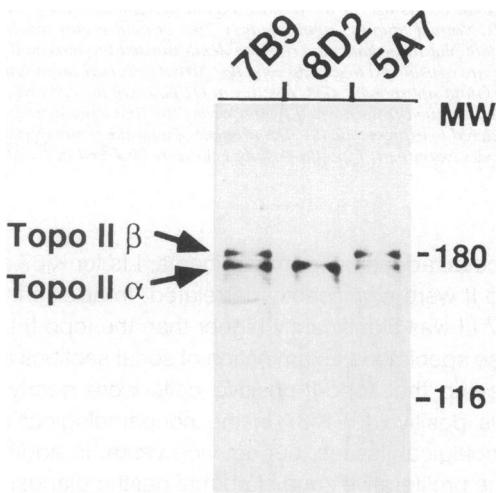

Figure 7. *Immunoblotting of human topo IIα and -β isoform.* Lane 1, *monoclonal antibody 7B9;* lane 2, *monoclonal antibody 8D2;* lane 3, *monoclonal antibody 5A7. Molecular weight markers (Bio-Rad) are indicated by an* arrow *in the left side of the membrane. In each lane, a protein extract containing approximately 50 μg of protein was used for analysis. After electrophoresis, the membrane was transferred. The membrane was incubated with the primary antibodies diluted at 1:100 for 15 hours at 4°C after blocking with 10% skim milk. The filter was incubated with horseradish-peroxidase-conjugated anti-mouse IgG, and the colorimetric reaction was performed with 2-chloronaphthol as a chromogen.*

Discussion

The immunolocalization of topo II has not been extensively studied in human tissues.[32] Topo II protein exists in mammalian cells in two distinct isoforms, ie, α and β. The α-isoform predominates in proliferating cells whereas the β-isoform predominates in resting cells.[33–35] We applied three different types of monoclonal antibodies, anti-α isotope, anti-β isotype, and anti-α and -β isotypes. We could detect topo II in tissue sections only by employing anti-α isotype and anti-α and -β isotypes antibodies. These findings of immunohistochemical staining for topo II were consistent with the immunochemical observation previously reported with human tissue[17,23] and with the fact that the level of expression of β-isotype of the enzyme is considered to be below the limit of detection by the immunostaining.

When pathologists evaluate cell proliferation in surgical pathology materials submitted to the laboratories by employing immunohistochemistry, at least the following two conditions need to be satisfied. First is the ability to perform immunostaining on routinely processed materials, ie, 10% formalin-fixed and paraffin-embedded tissue sections because these are the materials generally available in the pathology laboratory and used for histopathological diagnosis due to excellent morphological preservation. The second is that positively stained cells in tissue sections should represent proliferative cells. To meet the first condition, we employed 8% paraformaldehyde or 10% formalin as a fixative, performed immunostaining in both frozen and paraffin-embedded tissue sections, and compared the results. The patterns of immunolocalization of topo II in these two different fixatives and these two modes of tissue preparation were not different. In addition, there was a significantly positive correlation of topo II LI observed between frozen tissue sections and formalin-fixed, paraffin-embedded materials. Therefore, although autoclave pretreatment was required for antigen retrieval in paraffin-embedded tissue

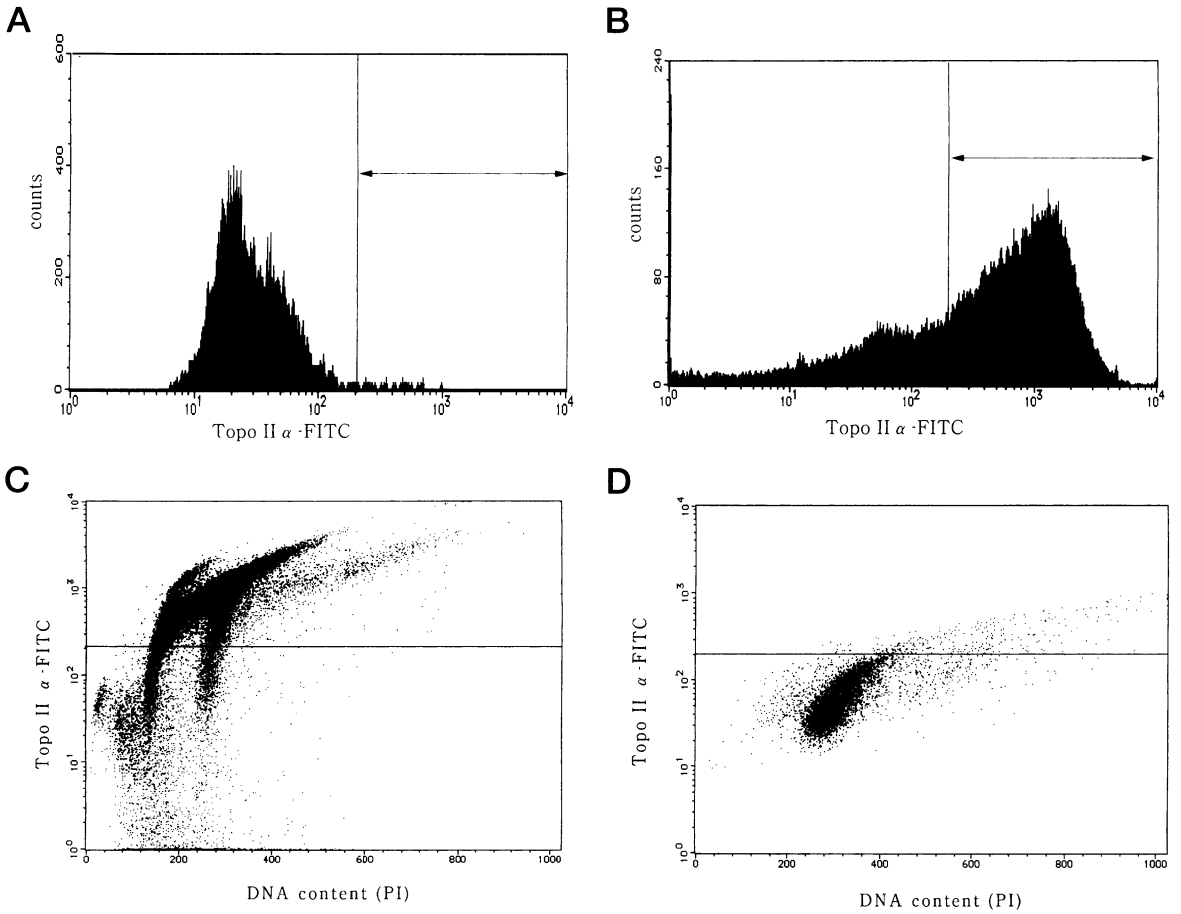

Figure 8. *Flow cytometric analysis of topo IIα and DNA content of Jurkat cells and the cells obtained from normal lymph nodes.* A: *Single-parameter fluorescent histogram of negative control aliquot in Jurkat cells; less than 2% of total events are in the positive region designated by the bar.* B: *Corresponding single-parameter fluorescent histogram of topo IIα anti-mouse FITC-stained aliquot; approximately 75% of total events are in the positive region designated by the bar.* C: *Topo IIα (FITC)* versus *DNA content (PI) dot plot in Jurkat cells. The line demonstrated the maximal level of fluorescence of negative control determined by* A *and* B. *The cells above this line are considered to express topo IIα. Jurkat cells had an aneuploid cell population and wide coefficient of variation in the topo-IIα-positive population (data not shown). Cells possibly at G1 phase of the cell cycle also expressed topo IIα.* D: *Topo IIα (FITC)* versus *DNA content (PI) dot plot in the cells obtained from normal lymph node. The line demonstrated the maximal level of fluorescence of negative control. The cells above this line are considered to express topo IIα. The number of topo-IIα-positive cells was 5% of total cells, due to the relatively small number of proliferative cells in lymph nodes examined. Topo-IIα-positive cells were observed in G2/M and S phases of the cell cycle.*

sections, we proved that immunohistochemical analysis of topo II can be performed on routinely processed surgical pathology materials.

A mouse monoclonal antibody, Ki67, has been frequently employed in the immunohistochemistry for the nuclear antigen.[8,9] However, the nature of nuclear antigens recognized by Ki67 has not been well characterized yet.[8,9] Proliferating cell nuclear antigen has also been widely used as an *in situ* marker of cell proliferation,[29] but its value has recently been questioned because it can be readily destroyed by prolonged fixation and can also be detected in cells not necessarily associated with cell proliferation after antigen retrieval procedures.[35,36] Therefore, to meet the second condition, we compared immunoreactivity for topo II with that for Ki67. In all non-neoplastic mucosa, adenoma, and carcinoma, LIs for Ki67 and topo II were significantly correlated. In addition, the Ki67 LI was significantly higher than the topo II LI in these specimens. Examination of serial sections also revealed that topo-II-positive cells were nearly always positive for Ki67 in the nonpathological and pathological tissues, but not vice versa. In addition, in the proliferative zone of normal gastric glands, the area of Ki67-positive cells was much wider than that of topo II. These data suggest that topo II immunohistochemistry can be useful for examining cell proliferation in routinely processed tissue sections. The present study evaluated topo II immunolocalization in human gastric tissues, including non-neoplastic mucosa, adenoma, and carcinoma. The topo II LI in adenomas and carcinomas of both the intestinal and

diffuse types was significantly higher than that in non-neoplastic gastric mucosa, which is consistent with previous findings of proliferative activity in human gastric neoplasms determined by Ki67 immunostaining.[30,37,38]

Among gastric neoplasms, the topo II LI of diffuse-type adenocarcinoma was significantly higher than that of adenoma ($P < 0.001$) or of intestinal-type adenocarcinoma ($P < 0.001$). There were, however, no significant differences in topo II LI between adenoma and intestinal-type adenocarcinoma. Similar findings were also obtained with Ki67 immunostaining. Little is known about cell proliferation in gastric adenomas, but these results indicate that there were no significant differences in cell proliferation between gastric adenomas and intestinal-type adenocarcinomas.

We evaluated the topo II/Ki67 index, ie, the topo II LI divided by Ki67 LI in the same areas of the lesions. The topo II/Ki67 index in gastric adenoma or carcinoma significantly exceeded that examined in non-neoplastic gastric mucosa. Among neoplastic lesions, the diffuse type had a significantly higher topo II/Ki67 index, but there were no significant differences in topo II/Ki67 index between adenomas and intestinal-type adenocarcinomas. Very recently, similar findings were also reported by Fogt et al.[39] In addition, results of two-parameter flow cytometry revealed that, in Jurkat cells, topo IIα expression was observed in cells at other than S and G2/M phases. Heck et al[14] demonstrated that the half-life of topo II is much shorter in normal cells than in transformed lymphoblastoid cells, which may explain the appearance of topo-II-positive G1 cells in the Jurkat cell line. These results all suggest that higher topo IIα expression in malignant cells reflects not only increased proliferative activity of these cells but also qualitative alteration of topo IIα expression associated with malignant transformation. As was described previously, topo II content in tumor cells is considered as a critical determinant for anti-cancer drug cytotoxic activity or drug sensitivity/resistance of various human tumors.[40] A number of studies correlated the level of topo II activity with response to topo-II-targeted drugs in various cancer cell lines.[40–43] In addition, not only quantitative but also qualitative changes of topo II in human cancer may be related to the sensitivity or resistance of topo-II-targeted anti-cancer chemotherapeutic agents.[40–43] Therefore, the topo II LI determined by immunohistochemistry in human malignancies may predict a sensitivity or resistance to topo-II-targeted chemotherapeutic agents, but additional investigations are required for clarification.

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