Jpn. J. Cancer Res. **90**, 758–764, July 1999

Indomethacin Suppresses the Growth of Colon 26, Meth-A and FM3A Tumors in Mice by Reducing the Prostaglandin E_2 Content and Telomerase Activity in Tumor Tissues

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The antitumor effect of indomethacin on Colon 26, Meth-A and FM3A tumors was investigated in mice. The prostaglandin E_2 content in tumor tissues was assayed to find out if indomethacin acts on tumors, and the telomerase activity in tumors and somatic tissues (testis, liver, spleen and colon) was also monitored during indomethacin treatment. Growth of Colon 26, Meth-A and FM3A tumors was significantly ($P<0.001–0.05$) suppressed by indomethacin compared to the untreated controls. The prostaglandin E_2 content in the three tumors was markedly ($P<0.001$) reduced by indomethacin. Telomerase activity in Colon 26 and FM3A tumors was significantly ($P<0.001$) lower than that of untreated tumors (80% and 45% decrease versus the controls, respectively), and the activity in Meth-A tumor was slightly decreased (10% decrease versus the control) by indomethacin. Telomerase activity in the somatic tissues was not significantly affected by indomethacin. In summary, this study shows the effectiveness of indomethacin as an antitumor agent against three types of tumors, and suggests that indomethacin affects telomerase activity in tumors *in vivo*.

Key words: Indomethacin — Prostaglandin E_2 — Telomerase activity — Fluorescence-based telomeric repeat amplification protocol

The antitumor effect of indomethacin on tumors in cancer patients[1,2] and in animals[3,4] has been described. We previously showed that indomethacin suppresses the growth of Colon 26 tumor in mice by enhancing the cellular immune function and improving cancer cachexia.[5] Indomethacin does not have a cytotoxic effect on Colon 26 tumor cells *in vitro*,[5] but it may have an antitumor effect via immunological pathways. We previously reported that indomethacin treatment of tumors reduces tumor size less in nude mice (deficient in T-lymphocytes) than in normal mice, compared with untreated tumors.[6] Although these results indicate that the effectiveness of indomethacin as an antitumor agent is, at least in part, due to its modification of T-cell-mediated immune functions, the precise mechanism of tumor growth suppression by indomethacin remains unknown.

Recent studies have shown that many proliferating tumor cells retain a certain level of telomerase activity.[7–9] We previously reported that tumor growth in Colon 26-bearing mice is significantly suppressed by indomethacin treatment compared with the untreated controls, and that the telomerase activity declines preferentially in tumor tissues, while telomerase activity in somatic tissues is not significantly affected by indomethacin treatment.[10] More recently, Kido *et al.*[11] reported that tumor size reduction by cisplatin treatment correlates with a decline in telomerase activity in tumor tissues.

In this study, we transplanted Colon 26, Meth-A and FM3A tumors into normal mice to assess if the effect of indomethacin is independent of tumor cell type. Prostaglandin E_2 synthesis is inhibited by indomethacin, and therefore we measured the prostaglandin E_2 content in tumors to confirm the effect of indomethacin on the tumor tissues. We examined the relationship between tumor growth and telomerase activity in tumor tissues, and whether the extent of tumor growth suppression correlates with a decline in telomerase activity in the tumors examined.

MATERIALS AND METHODS

Cell lines, host and tumor transplantation Colon 26 (murine colon tumor), Meth-A (murine fibrosarcoma) and FM3A (murine breast tumor) cell lines were kindly donated by Kyowa Hakko, Tokyo. A cell suspension containing 2.5×10^6 cells of each line in physiological saline (0.025 ml) was transplanted into the right hind foot pads (day 0) of experimental mice. Colon 26 tumor was transplanted into 6-week-old male CDF_1 mice (Charles River,

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Kanagawa); Meth-A tumor was transplanted into 6-week-old male BALB/c mice (Charles River); and FM3A tumor was transplanted into 6-week-old male C3H/He mice (Charles River). All the mice were cared for according to international standards (EU and NIH criteria).

Drug preparation and administration Indomethacin crystals (Sigma, St. Louis, MO) were mixed with a 0.5% carboxymethylcellulose (CMC; Sigma) solution and the mixture was adjusted to 1.0 mg/kg body weight/0.2 ml of 0.5% CMC. The mice were divided into two groups for each tumor cell line: an untreated control group and a group treated with indomethacin. Indomethacin in CMC solution was given by oral gavage for two weeks from day 7 to the tumor-bearing mice at a dose of 1.0 mg/kg body weight twice a day (total dose; 2.0 mg/kg body weight per day), and 0.2 ml of 0.5% CMC solution without indomethacin was given by oral gavage to the untreated control tumor-bearing mice in the same way. Both untreated control and indomethacin-treated groups consisted of seven randomly assigned mice for each tumor. All mice were given a standard diet and tap water freely.

***In vitro* cytotoxicity of indomethacin on three tumor cell lines** In an *in vitro* study, we evaluated the IC_{50} of indomethacin in the three tumor cell lines using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay, as reported by Ogino and Hanazono,[12] using a slight modification of the method reported by Mosmann.[13]

Measurement of tumor weight In a preliminary study, we first assessed the correlation between tumor weight and estimated tumor volume (TV). Tumor length (*a*: cm) and tumor width (*b*: cm) were measured externally at random times after tumor transplantation, and the TV was calculated as $ab^2/2$. Then, the mice were deeply anesthetized with an excessive dose of pentobarbital and killed. Both right (A: with a tumor) and left (B: tumor-free) hind foot pads were dissected and weighed. The difference between A and B was taken as the tumor weight (TW), which was calculated by the formula: TW=0.8266 TV+0.4135 (g), as reported by Ogino *et al*.[5] In this way, tumor weight was measured at 2- or 3-day intervals from day 7 in both untreated and indomethacin-treated mice for each tumor. The results were expressed as the mean g±SEM. The experiment was stopped on day 21, when the mean tumor weight in the untreated control mice reached approximately 15% of the body weight of normal mice of the same age. On day 21 (9 weeks of age), all mice in each group were killed by cervical dislocation, and the tumors, testes, liver, spleen, colon and other somatic tissues were dissected. Similarly, tumor-free 9-week-old male CDF_1 mice (n=5), BALB/c mice (n=5) and C3H/He mice (n=5) were killed by cervical dislocation, and the testes, liver, spleen, colon and other somatic tissues were dissected. The tumor tissues and somatic tissues obtained were immediately frozen in liquid nitrogen, and were stored at −85°C until required for analysis of the telomerase activity.

Measurement of prostaglandin E_2 content in the tumor tissues Some fragments of the tumor tissues were cut from the whole tumor and were thoroughly rinsed in physiological saline containing ethylenediaminetetraacetic acid (Sigma, 10 m*M*) and indomethacin (Sigma, 0.1 m*M*). Then they were immediately frozen in liquid nitrogen, and stored at −85°C until required for measurement of the prostaglandin E_2 content in the tumor tissues. The tumor tissues were homogenized in ethanol, and mixed by vortexing, then the prostaglandins were extracted. The extract was subjected to silicic acid column chromatography (0.6 g, 100 mesh; Mallinkrodt Baker, Phillipsburg, NJ) to separate prostaglandin E_2, as reported by Ogino *et al*.[14] An aliquot of eluates in the prostaglandin E_2 fraction was used to measure prostaglandin E_2, using a standard radioimmunoassay kit (DuPont-New England Nuclear, Boston, MA). The results were expressed both as the mean pg/mg wet weight±SEM and the mean pg/mg protein± SEM (n=5). The tissue protein was determined by Lowry's method.[15]

Detection and measurement of telomerase activity The telomerase activity was detected and measured using a semi-quantitative, fluorescence-based telomeric repeat amplification protocol method (F-TRAP) with a "TRAP-eze" Telomerase Detection Kit (ONCOR®, Gaithersburg, MD), as reported by Hisatomi *et al*.[16] The tumor tissues and other somatic tissues (50 mg wet weight of each tissue) and the testes (the whole tissues) were briefly homogenized in 200 μl of ice-chilled CHAPS Lysis Buffer (TRAP-eze) and incubated for 30 min on ice. After incubation, the lysates were centrifuged at 12,000*g* for 20 min at 4°C. The supernatants were rapidly frozen and kept at −85°C. The protein concentration in the tissue samples was determined using "Coomassie" Protein Assay Reagent (Pierce, Rockford, IL), and an aliquot of extract containing 1.0 μg of protein was used as an enzyme source for each TRAP assay according to the manufacturer's instructions. Aliquots of the extract were incubated with 0.1 ng of Cy-5 labeled TS primer (5′-AATCCGTCGAGCAGAGTT-3′) in Master Mix (TRAP-eze). After a 30 min incubation at 30°C, polymerase chain reaction (PCR) was run at 94°C/30 s, 60°C/30 s and 72°C/45 s for 30 cycles. An external control (TSR8: TRAP-eze) was used as a positive control and CHAPS Lysis Buffer (TRAP-eze) was used as a negative control. The products were diluted with an equal volume of formamide dye solution and heated for 5 min at 94°C. Then they were applied (5 μl/lane) to a 10% denaturing gel containing 6 *M* urea in an automated DNA sequencer (ALF red DNA sequencer: Amersham Pharmacia Biotech, Buckinghamshire, UK). During electrophoresis at 45 W, the temperature of the gel was kept at 45°C.

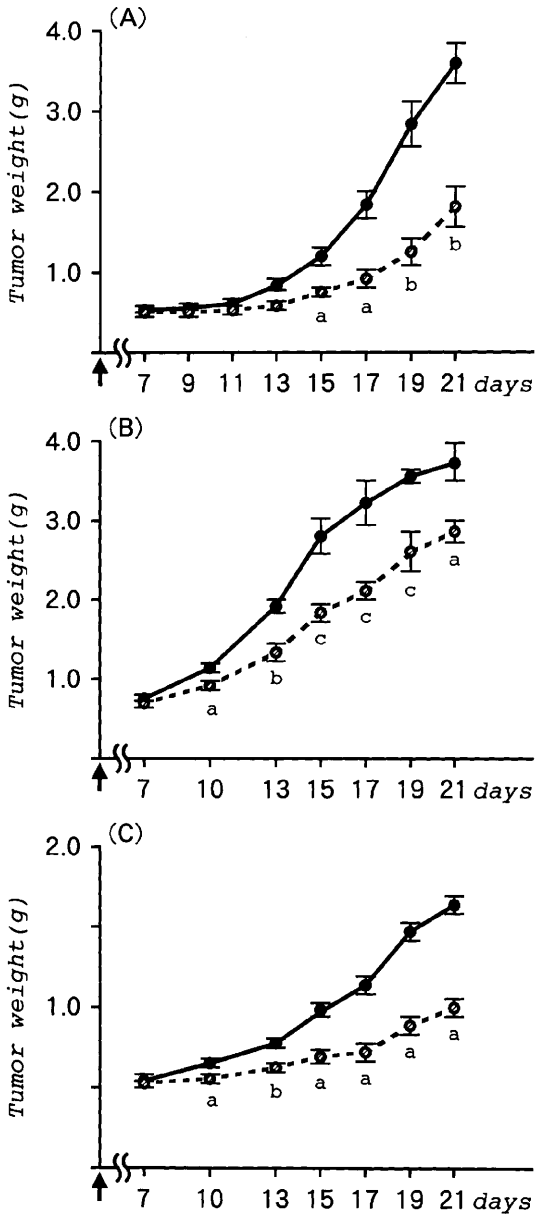

Fig. 1. Tumor weight in (A) Colon 26-bearing CDF_1 mice; (B) Meth-A-bearing BALB/c mice; and (C) FM3A-bearing C3H/He mice. The tumor weight in each group is expressed as the mean g±SEM for seven mice. Untreated control groups, closed circles with solid lines; indomethacin-treated groups, open circles with broken lines. Untreated group vs. indomethacin-treated group: (A) $^aP<0.01$, $^bP<0.001$; (B) $^aP<0.05$, $^bP<0.001$, $^cP<0.01$; (C) $^aP<0.001$, $^bP<0.01$.

The data obtained from the sequencer were collected and automatically analyzed using Allele Links software (Amersham Pharmacia Biotech). The area of the peak of each hexanucleotide pattern was quantified. The telomerase activity was calculated by using the following formula according to the manufacturer's instructions:

$$\text{TPG (total product generated) units} = [A \times B^{-1}/C \times D^{-1}] \times 100$$

where, *A*: measured total area of telomerase activity (50, 56 , 62 , 68 bp...)
B: measured area of the internal control (36 bp)
C: measured total area of telomerase activity (50, 56, 62, 68 bp...) in the external control
D: measured area of the internal control (36 bp) in the external control

The results were expressed as the mean TPG units/μg protein±SEM.

Statistical analysis Statistical analysis was made using a two-tailed *t*-test. A difference was considered statistically significant when the *P* value was less than 0.05.

RESULTS

Tumor growth Tumor growth of Colon 26 in CDF_1 mice treated with indomethacin was significantly reduced from day 15 ($P<0.001-0.01$ vs. controls; Fig. 1A). The mean tumor weight was suppressed by indomethacin treatment, i.e. a maximal 55% reduction versus the control. Tumor growth of Meth-A in BALB/c mice treated with indomethacin was significantly reduced from day 10 ($P<0.001-0.05$ vs. controls; Fig. 1B). The mean tumor weight was suppressed by indomethacin treatment, i.e. a maximal 35% reduction versus the control. Tumor growth of FM3A in C3H/He mice treated with indomethacin was significantly reduced from day 10 ($P<0.001-0.01$ vs. control; Fig. 1C). The mean tumor weight was suppressed by indomethacin treatment, i.e. a maximal 40% reduction versus the control.

IC_{50} of indomethacin in three tumor cell lines The IC_{50} of indomethacin in Colon 26 tumor was 93.4±1.6 (the mean μg/ml±SEM of five different experiments each done in duplicate), but the IC_{50} of indomethacin in Meth-A and FM3A tumors was higher than 100 μg/ml.

Prostaglandin E_2 content in the tumor tissues The tissue content of prostaglandin E_2 was markedly and significantly ($P<0.001$ vs. controls) reduced in Colon 26, Meth-A and FM3A tumors treated with indomethacin, i.e. an approximately 90% reduction versus the untreated controls (Table I).

Telomerase activity The telomerase activity in Colon 26 tumor tissues was significantly decreased ($P<0.001$ vs. control) by indomethacin treatment compared with the untreated controls (Table II), i.e., an approximately 80% decrease. The number and peak area of TRAP products in a typical electrophoresis pattern were decreased by indomethacin treatment (lane 2 in Fig. 2a) compared with the untreated control (lane 1 in Fig. 2a). Meth-A tumor tissues expressed less telomerase activity than the cancer

Table I. Prostaglandin E_2 Content in the Tumor Tissues

		pg/mg wet weight	pg/mg protein
Colon 26	Untreated control tumors (n=5)	48.1±2.6	456.6±21.1
	Indomethacin-treated tumors (n=5)	4.8±0.4	48.2±5.5
Meth-A	Untreated control tumors (n=5)	259.3±12.8	1853.8±39.8
	Indomethacin-treated tumors (n=5)	15.8±1.2	121.6±8.2
FM3A	Untreated control tumors (n=5)	662.7±21.4	4465.0±159.1
	Indomethacin-treated tumors (n=5)	50.6±5.2	362.9±47.6

Data are the mean pg/mg wet weight and pg/mg protein±SEM values.
Prostaglandin E_2 content in the tumor tissues was significantly (P<0.001) reduced by indomethacin treatment compared with the untreated controls.

Table II. Telomerase Activity in the Tumor Tissues

	Colon 26	Meth-A	FM3A
Untreated control tumors (n=7)	176.58±2.31[a)]	98.14±10.66[b)]	205.01±9.35[c)]
Indomethacin-treated tumors (n=7)	34.21±0.95[a)]	88.04±7.53[b)]	114.56±8.86[c)]

Data are the mean TPG units/μg protein±SEM values.
a) P<0.001, *c*) P<0.001, *b*) no significant difference: telomerase activity in the untreated control tumors vs. the indomethacin-treated tumors.

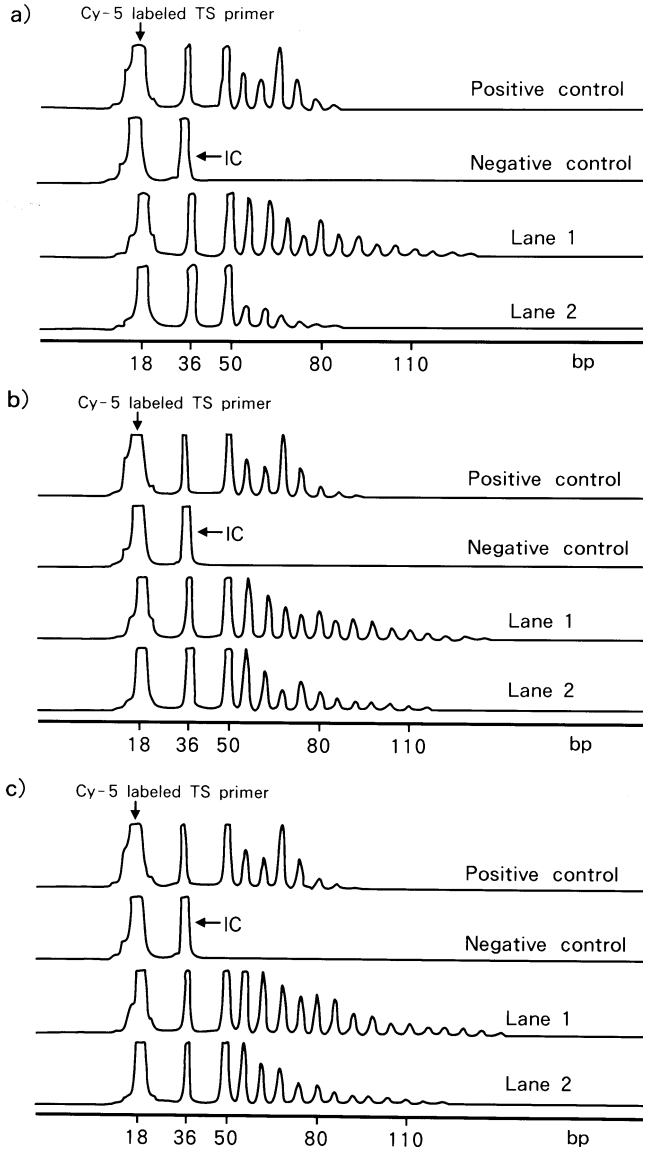

Fig. 2. Typical electrophoresis pattern of TRAP products scanned by an ALF red DNA sequencer in (a) Colon 26 tumor in CDF_1 mice; (b) Meth-A tumor in BALB/c mice; and (c) FM3A tumor in C3H/He mice. The first peak is a Cy-5 labeled TS primer. The second peak is an internal control (IC: 36 bp ITAS). The PCR products of the telomerase extension yielded successive hexanucleotide peaks (50, 56, 62, 68, 74 bp ....) from the third peak (50 bp), which is the first amplifiable product. From the top downwards: positive control (TSR 8); negative control (CHAPS Lysis buffer); lane 1, tumor in the untreated control mice; lane 2, tumor treated with indomethacin.

cells, i.e. approximately one-half that of the cancer cells. The mean telomerase activity in Meth-A tumor tissues was slightly, though not significantly, decreased by indomethacin treatment (about 10% decrease; Table II). Fig. 2b shows the electrophoresis pattern of TRAP products (lane 1, untreated control; lane 2, indomethacin-treated tumor). The telomerase activity in FM3A tumor tissues was significantly reduced (P<0.001 vs. control) by indomethacin treatment compared with the untreated control (about 45% decrease; Table II). Fig. 2c shows the electrophoresis pattern of TRAP products (lane 1, untreated control; lane 2, indomethacin-treated tumor).

In both CDF_1 and BALB/c mice, telomerase activity was detected in the testis, liver and colon (Table III), but not in the spleen. However, telomerase activity was detected in the spleen in C3H/He mice (Table III). In both the tumor-free group and the group with tumors of the three mouse strains, telomerase activity was also detected in other somatic tissues, but at much lower levels (data not shown). Although the basal levels of telomerase activity in the somatic tissues varied among the mouse strains, the

Table III. Telomerase Activity in the Somatic Tissues for Each Mouse Strain

		Testis	Liver	Spleen	Colon
Colon 26	Untreated control mice (n=7)	91.97±3.24	49.16±0.75	ND	31.00±1.28
	Indomethacin-treated mice (n=7)	82.16±1.73	48.64±0.42	ND	28.17±3.79
	Tumor non-bearing mice (CDF_1: n=5)	84.93±1.15	48.15±0.56	ND	32.76±1.38
Meth-A	Untreated control mice (n=7)	132.75±4.19	48.53±2.90	ND	33.68±3.41
	Indomethacin-treated mice (n=7)	122.75±9.26	47.84±3.86	ND	34.18±3.38
	Tumor non-bearing mice (BALB/c: n=5)	127.25±10.26	49.60±0.56	ND	31.63±9.28
FM3A	Untreated control mice (n=7)	35.17±3.10	56.46±1.50	39.23±5.54	31.00±1.82
	Indomethacin-treated mice (n=7)	29.33±2.50	53.68±1.15	41.36±3.96	28.71±3.97
	Tumor non-bearing mice (C3H/He: n=5)	33.94±4.18	56.40±3.96	42.80±5.62	32.67±1.83

Data are the mean TPG units/μg protein±SEM values.
ND; not detected.
No significant differences among the untreated control mice, indomethacin-treated mice and mice without tumors in the somatic tissues for each mouse strain.

telomerase activity in the somatic tissues of the untreated control mice with tumor, mice with tumor treated with indomethacin and mice without tumor showed no significant differences.

DISCUSSION

In this study, we observed that indomethacin treatment suppressed the growth of all tumors examined. We compared the inhibitory effect of indomethacin on tumor growth with the reported inhibitory effects of other chemotherapeutic agents: the maximal reduction of 55% in Colon-26 tumor size of our study is comparable with the antitumor effect of cisplatin,[17] as well as the antitumor effect of 5-fluorouracil alone in Meth-A tumor-bearing mice,[18] and the maximal tumor size reduction of 40% in the FM3A tumor in our study is comparable with the antitumor effect of bleomycin[19] or UFT (a fluorouracil compound)[20] alone. However, in our *in vitro* study, the concentration equivalent to the achievable maximal plasma concentration (MPC), which is a little higher than 1.0 μg/ml,[21] did not show a cytotoxic effect on Colon 26, Meth-A and FM3A tumor cells, as judged from the IC_{50} values of indomethacin in these tumor cells using MTT assay. The IC_{50} values of indomethacin in the three types of tumor cells were about 100-fold higher than the MPC *in vivo*. Maca[22] reported a similar disparity of the effect of indomethacin between *in vitro* and *in vivo* studies on Lewis lung carcinoma transplanted into normal, nude and beige mice. We conclude that indomethacin treatment *in vivo* does not directly inhibit tumor growth, irrespective of the tumor type or mouse strain used.

Prostaglandin E_2, which was produced by all tumors examined, regulates cytokine production in inflammatory cells[23, 24] and modifies cellular immune responses[25, 26]; an exogenously added prostaglandin E_2 analogue (16,16-dimethylprostaglandin E_2 methyl ester) is a general immunosuppressant *in vivo*.[27] Therefore, the effect of indomethacin on the tumor tissues is, at least in part, due to the inhibition of the increased formation of immunosuppressing prostaglandin E_2. We also showed that prolonged treatment with indomethacin resulted in a marked reduction of prostaglandin E_2 production in the three types of tumors. This study suggests the involvement of prostaglandin E_2 in enhancing tumor cell proliferation as a bioactive modulator in addition to its immunosuppressing effect, as suggested in previous reports.[28, 29]

Telomerase activity is an *in vitro* marker of the cytotoxicity of chemotherapeutic agents,[30] and telomerase activity positively correlates with the growth of tumors treated by cisplatin *in vivo*.[12] In this study, we showed that a decline in telomerase activity might correlate with tumor size reduction by indomethacin treatment *in vivo*, because the order of the maximal tumor size reduction (Colon 26 (55%) > FM3A (40%) > Meth-A (35%)) agreed with the order of mean inhibition of telomerase activity (Colon 26 (80%) > FM3A (45%) > Meth-A (10%)). However, indomethacin treatment did not significantly decrease the telomerase activity in Meth-A tumor despite its significant suppressive effect on tumor growth. This effect of indomethacin treatment on telomerase activity in tumor tissues *in vivo* has not been reported before. In addition, the fact that telomerase activity in the testis, liver, spleen and colon was not significantly affected by indomethacin treatment is of great interest. These results suggest that indomethacin does not directly inhibit telomerase activity.

From these findings, we hypothesize that the decline in telomerase activity in tumors treated with indomethacin *in vivo* involves the following mechanisms: a decrease of vascular permeability by inhibition of the local production

of prostaglandin E_2, which is generated in tumors and in inflammatory cells,[31, 32] leading to suppression of tumor growth through a decrease of nutrition; secondly, an enhancement of immune response via inhibition of increased formation of immunosuppressing prostaglandin E_2.

Although we did not find a definite relationship between the extent of tumor growth reduction and the preferential decline in telomerase activity in tumor tissues by indomethacin treatment, we confirmed the effectiveness of indomethacin as an antitumor agent for three types of murine malignant tumors. Indomethacin also reduces large bowel carcinogenesis induced by acetoxymethylmethylnitrosamine[33] and modifies uterine cervical carcinogenesis induced by methylanthracene,[34] and prolonged indomethacin therapy reduces the development of spontaneous mammary tumors and suppresses cancer progression.[35] Considering that indomethacin has been used widely and safely for a long time, it should be useful not only as a chemopreventive agent, but also as an antitumor agent enhancing T-cell-mediated immune function by regulating prostaglandin E_2 biosynthesis in tumor cells and/or inflammatory cells.

ACKNOWLEDGMENTS

We are indebted to Prof. Toshiko Fujii at the Department of Pharmacology and Prof. Takashi Nakamura at the 3rd Department of Internal Medicine, Teikyo University School of Medicine, for their continuous encouragement and valuable advice. We thank Kyowa Hakko, Tokyo, for providing tumor cell lines, and Mr. Christopher Moreby for help in preparing the manuscript.

(Received March 8, 1999/Revised April 22, 1999/Accepted April 28, 1999)

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