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## INHIBITORY EFFECTS OF INDOMETHACIN ON GROWTH AND PROLIFERATION OF GASTRIC CARCINOMA CELLS KATO III.

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The effects of indomethacin on growth and proliferation of gastric carcinoma cells KATO III were examined. Indomethacin ( $10^{-4}$  and  $10^{-3}$ M) significantly inhibited cell growth and these effects were not affected by treatment with 16,16-dimethyl prostaglandin E<sub>2</sub> ( $3 \times 10^{-7}$ M and  $3 \times 10^{-6}$ M). Indomethacin  $10^{-3}$ M significantly reduced cell viability and completely inhibited cell growth. Indomethacin  $10^{-4}$ M did not affect cell viability and its inhibitory effect of cell growth became apparent on the fifth day of culture. Indomethacin  $10^{-4}$ M reduced BrdU labeling index within 2 hours. These results suggest that indomethacin inhibited growth and proliferation of gastric carcinoma cells KATO III. This effect is not mediated by prostaglandins.

**Key words:** Kato III carcinoma cells, proliferation, indomethacin

### INTRODUCTION

A continuous cell renewal and proliferation is one of the major defensive mechanisms of the gastric mucosa responsible for maintenance of mucosal integrity and enabling repair of injury and ulcer healing (1). Nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin cause gastric mucosal damage (2) and delay ulcer healing (3). The precise mechanisms of these effects are not clear. Recent studies (4—9) suggest that effect of NSAIDs on gastric epithelial cell proliferation may be in part responsible for these mechanisms. There are no studies regarding effects of NSAIDs on cell proliferation in gastric epithelial cultured cells. In this paper, we examined effects of indomethacin on growth and proliferation of gastric carcinoma cells KATO III.

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## MATERIALS AND METHODS

*Drugs*

Indomethacin (Sigma Chemical Co., St. Louis, MO.) was dissolved in 200 mM  $\text{Na}_2\text{CO}_3$ , adjusted to pH 7.4 with 200 mM  $\text{NaH}_2\text{PO}_4$ . 16,16-dimethyl prostaglandin  $\text{E}_2$  (dmPGE<sub>2</sub>; Cayman Chemical Co., Ann Arbor, MI.) was dissolved in absolute ethanol and diluted with RPMI 1640 medium.

*Cell culture and cell growth experiment*

Gastric carcinoma cells KATO III were obtained from American Type Culture Collection and cultured in RPMI 1640 medium containing 20% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) at 37°C with 5%  $\text{CO}_2$  in air in a humidified atmosphere. After the culture, cells were inoculated in 24 wells multiplate adjusted to  $2 \times 10^4$  cells/well in RPMI medium containing 10% FBS and the antibiotics described above. Various dose of indomethacin or dmPGE<sub>2</sub> were added to the culture at the beginning of cell growth (DAY 0). Number of cells were counted in a hemocytometer on the first, third, fifth, and seventh day of culture.

*Cell viability and experimental design*

Aliquots of KATO III cell suspension were incubated in oxygenated KH medium containing  $10^{-5}$ ,  $10^{-4}$ , and  $10^{-3}$  M indomethacin at 37°C for 2 hours and then cell viability was assessed by fast green exclusion test according to the method described previously (10). In brief, 16  $\mu\text{l}$  of 2% fast green (Sigma) were added to 0.2 ml cell suspension. After shaking gently, cell suspension was poured into the chamber of Cytospin centrifuge (Shandon Southern Instruments Inc., Sewickley, PA) and centrifuge at 600 rpm for 3 minutes. The resulting slides were then counterstained with hematoxylin and eosin. The viability was expressed as the percentage of viable cells in 10 microscopic field.

*BrdU staining*

Aliquots of KATO III cell suspension were incubated in KH medium containing  $10^{-4}$  M indomethacin or vehicle at 37°C for 2 hours and then 1 mM of 5-bromo-2'-deoxyuridine (BrdU; Sigma) were added to the medium and incubated for 1 hour. Sample slides were made by Cytospin centrifuge as described above. Cells were fixed in cold acetone. Endogenous peroxidase was quenched with 3%  $\text{H}_2\text{O}_2$  for 20 minutes. The slides were washed with distilled water and incubated in 4N HCl for 20 minutes at room temperature to denature double-stranded DNA and rinsed with 0.1 M  $\text{Na}_2\text{B}_4\text{O}_7$  (pH 8.5) for 5 minutes to neutralize the acid, and washed with distilled water and phosphate buffer saline (PBS). Cells were incubated with monoclonal mouse anti-BrdU antibody (Dako Corporation, Carpinteria, CA), diluted 1:100 in PBS containing 1% BSA for 1 hour and washed with PBS. Slides were incubated with goat biotinylated anti-mouse IgG (diluted 1:20 in PBS; Sigma) for 30 minutes and rinsed with PBS twice and covered with extravidin-peroxidase conjugate (diluted 1:20 in PBS; Sigma) for 20 minutes. Slides were stained with 3-amino-9-ethyl-carbazole (Sigma). Light counterstaining with hematoxylin was used to visualize nuclei. BrdU labeling index was defined as the percentage of labeled cells in relation to the 500 cells.

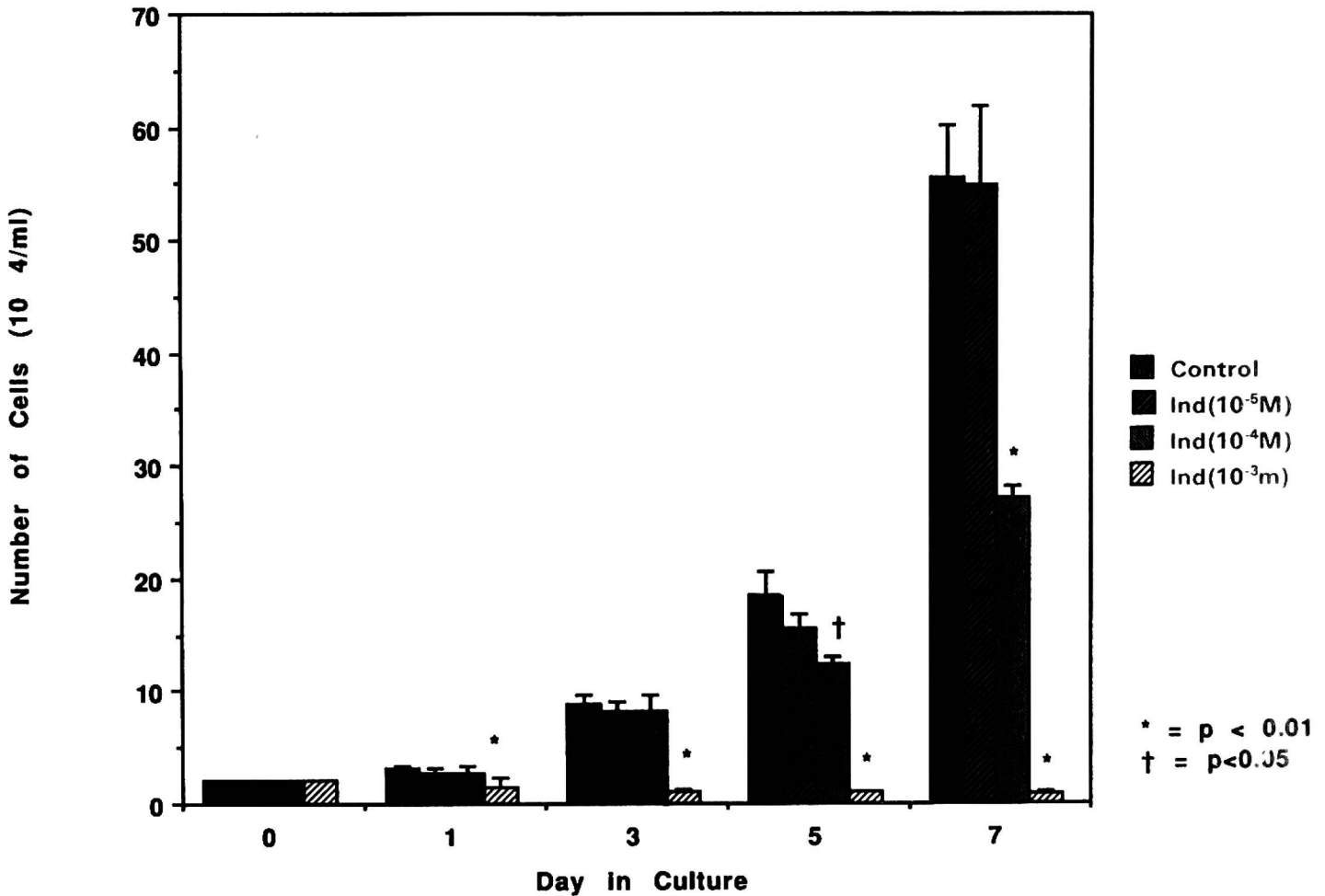
*Statistical analysis*

Student's unpaired t-test was used for statistical analysis. Data were expressed as mean  $\pm$  SE.

## RESULTS

*Effect of indomethacin on cell growth*

Indomethacin  $10^{-4}\text{M}$  significantly inhibited growth of gastric carcinoma cells KATO III on the fifth and seventh days of culture and in the groups treated with indomethacin  $10^{-3}\text{M}$  no cell growth occurred (*Fig. 1*).  $\text{dmPGE}_2$



*Fig 1.* Effects of indomethacin on the growth of KATO III cells. KATO III cells ( $2 \times 10^4$  cells/well) were cultured for various periods in RPMI 1640 medium containing 10% FBS with indomethacin  $10^{-5}\text{M}$ ,  $10^{-4}\text{M}$ ,  $10^{-3}\text{M}$ , or vehicle. Various dose of indomethacin were added to medium on the beginning of culture. Data are mean  $\pm$  SE. \*  $p < 0.01$  vs. vehicle-treated group for the same day.

$3 \times 10^{-6}\text{M}$  inhibited, to a lesser degree compared to indomethacin-treated group, cell growth of KATO III on the seventh days of culture.  $\text{dmPGE}_2$  ( $3 \times 10^{-7}\text{M}$  and  $3 \times 10^{-6}\text{M}$ ) did not affect the inhibition of cell growth by indomethacin on the seventh day of culture (*Fig. 2*).

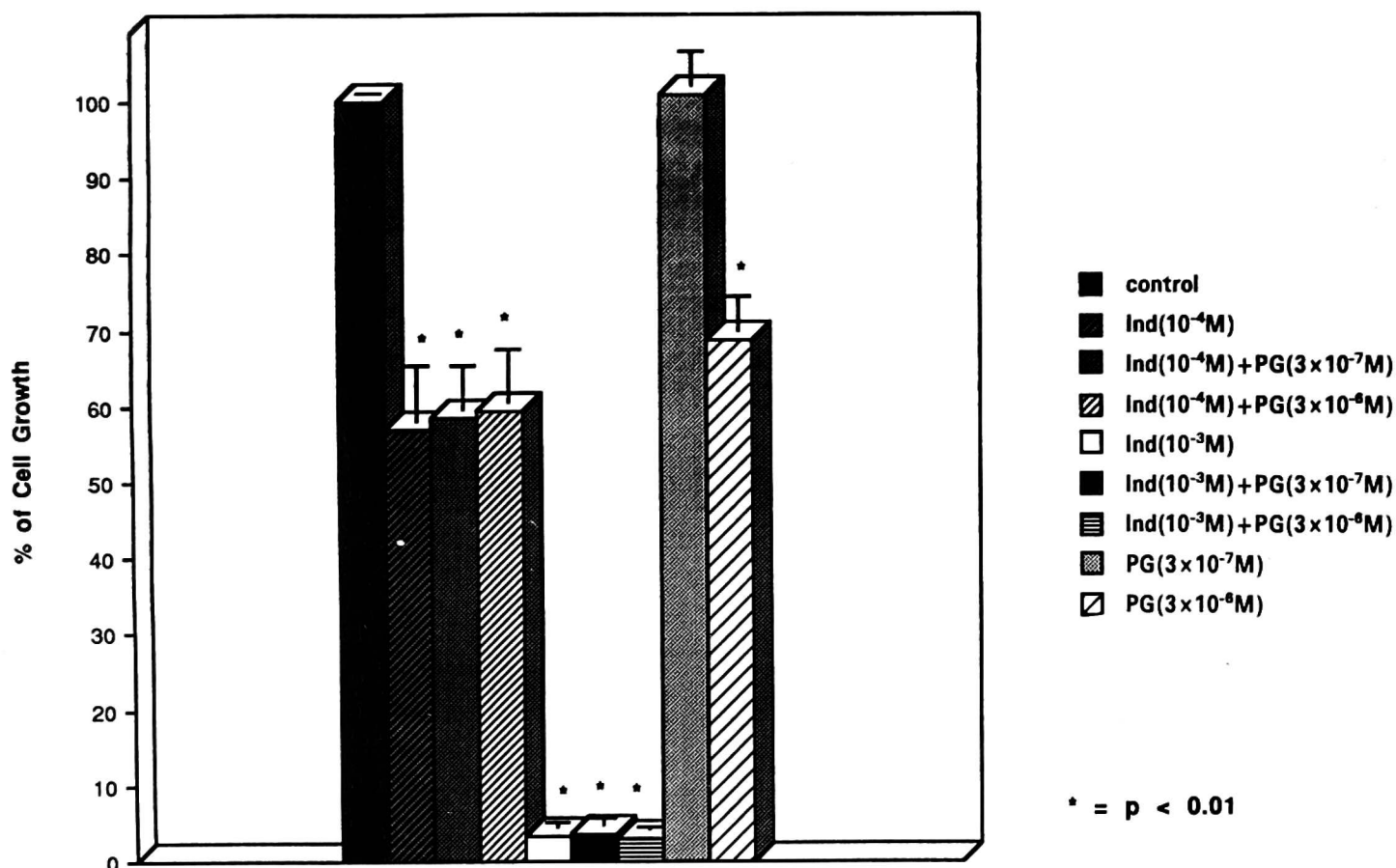
*Effect of indomethacin on cell injury*

Effects of various doses of indomethacin on cell viability after 2 hours incubation are shown in *Table 1*. Indomethacin  $10^{-3}\text{M}$  reduced cell viability

significantly, whereas indomethacin  $10^{-4}$  and  $10^{-5}$ M did not affect the cell viability compared to the vehicle-treated group.

*Table 1.* Effects of indomethacin on viability of KATO III cell. Cells were incubated with KH medium containing various dose of indomethacin for 2 hours. Cell viability was assessed by fast green dye exclusion test. Data are mean  $\pm$  SE. \* $p < 0.01$  vs. control.

Treatment	Cell Viability
control	$91.6 \pm 2.8$
indomethacin $10^{-5}$ M	$90.7 \pm 1.0$
$10^{-4}$ M	$91.2 \pm 1.2$
$10^{-3}$ M	$78.2 \pm 3.9^*$



*Fig 2.* Effects of 16,16-dimethyl prostaglandin  $E_2$  on indomethacin-induced inhibition of the growth of KATO III cells. Cells were counted on the seventh day of culture. The data are expressed as percentage of the control value. Data are mean  $\pm$  SE. \* $p < 0.01$  vs. control.

### *Effect of indomethacin on cell proliferation*

Incubation of cells with indomethacin  $10^{-4}$ M for 2 hours significantly reduced BrdU labeling index compared to the vehicle-treated group (*Table 2*).

Table 2. Effects of indomethacin on BrdU labeling index. KATO III cells were incubated with indomethacin  $10^{-4}\text{M}$  and then 1 mM BrdU was added. BrdU labeling index is expressed as percentage of labeled cells. Data are mean  $\pm$ SE. \* $p < 0.01$  vs. vehicle-treated group.

Treatment	BrdU LI (%)
Vehicle	$19.8 \pm 3.0$
indomethacin ( $10^{-4}\text{M}$ )	$10.3 \pm 3.6^*$

## DISCUSSION

Chronic ingestion of NSAIDs stimulates gastric mucosal cell proliferation in humans (5, 7) and rats (4—6). In gastric ulcers, NSAIDs inhibit cell proliferation at ulcer edge (8, 9) and this effect is reported to be reversed by co-administration of exogenous prostaglandins (9). These effects could explain, in part, adaptation of gastric mucosa by continuous ingestion of NSAIDs and epidemiologic feature of gastric ulcer in patients taking long-term NSAIDs therapy. The precise mechanism of these effects is not clear yet and experiments of NSAIDs on cell proliferation *in vitro* may explain this mechanism. There are, however, no studies using normal pure gastric cultured cells. In our study, we examined effects of indomethacin on growth and proliferation of gastric cultured KATO III cells derived from a human gastric carcinoma, which are classified as mucus producing signet ring cells (11) and considered to originate from the germinal region of the gastric gland (12).

We demonstrated that indomethacin inhibited growth and proliferation of gastric carcinoma cells KATO III. Indomethacin  $10^{-4}\text{M}$ , a dose which did not affect cell viability, inhibited cell growth on the fifth and seventh days of culture and inhibited cell proliferation, whereas indomethacin  $10^{-3}\text{M}$  (which caused cell injury within 2 hours) inhibited completely cell growth. These results mean that inhibition of cell growth by high dose ( $10^{-3}\text{M}$ ) of indomethacin was in part due to its immediate toxic action, but that of indomethacin  $10^{-4}\text{M}$  was related to the mechanisms without injurious action. In other cell preparation such as rat hepatoma and human fibroblast (13, 14), indomethacin ( $4 \times 10^{-3}\text{M}$  which did not impair cell viability) inhibited cell proliferation arresting cells in G1 phase. Thus one possibility of the mechanism of inhibition of cell growth is that indomethacin may affect the cell cycle.

Another interesting result was that inhibition of cell growth by indomethacin  $10^{-4}\text{M}$  became apparent from the fifth day of culture although indomethacin was added to the medium on the day of initial inoculation. In

recent study, Nakamura *et al.* (15, 16) demonstrated that in KATO III cells only a single addition of various PGs was able to induce inhibition of cell growth on the sixth day of culture and this effect was due to binding guanine nucleotide-binding stimulatory protein (Gs), stimulating adenylate cyclase activity and accumulating cyclic adenosine monophosphate (cAMP) production. Our data revealed that dmPGE<sub>2</sub> inhibited cell growth but to a lesser degree than the indomethacin-treated group and dmPGE<sub>2</sub> did not affect inhibition of cell growth by indomethacin. Therefore mechanisms of inhibitory effects by indomethacin may be different from that of PGs and at least not related to endogenous PGs.

However, Nakamura *et al.* (16) also demonstrated that neither PGE<sub>1</sub>, PGE<sub>2</sub>, nor PGF<sub>2α</sub> was detected in the medium during 24 hour culture of KATO III cells either in the presence or in the absence of indomethacin. PGs are synthesized in normal gastric mucosal cells and play a crucial role in the gastric mucosal defense. KATO III cells may not reflect the normal gastric epithelial cell physiology and examination of normal gastric cultured cell is needed to explain the mechanism of cell proliferation in gastric mucosa.

In summary, this study demonstrated that indomethacin inhibited growth and proliferation of gastric KATO III cells independently of PGs.

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