

# Synergistic Effect of Indomethacin and Bleomycin on Tumor Growth Produced by Activating Antitumor Immunity

Ryo Matsushita,<sup>1</sup> Kenji Hattori,<sup>2</sup> Kaori Hayashi,<sup>3</sup> Hisashi Iizasa,<sup>2</sup> Fujio Ichimura,<sup>4</sup> and Emi Nakashima<sup>2,5</sup>

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## INTRODUCTION

Cancer cells and macrophages produce large amounts of prostaglandin (PG) E<sub>2</sub>, which suppresses the cellular immune reaction in tumor-bearing animals (1). These findings suggest that an inhibitor of PG synthesis might be able to restore immune activity against tumors. It has been reported that indomethacin (IND) inhibits tumor growth when administered on its own and exhibits synergistic effects with several other biological response-modifiers in transplanted tumor models (2–4). In clinical studies, it has been reported that anti-inflammatory treatment may prolong the survival of undernourished patients with metastatic solid tumors (5).

In addition, several studies have shown that antitumor drugs restore cellular immune responses. In one case, bleomycin (BLM) increased gamma interferon, tumor necrosis factor alpha, and nitric oxide production by macrophages (6).

The above reports indicate that a marked synergistic effect on tumor growth is likely to be observed when IND and BLM are coadministered. To examine whether the tumor-suppressive effect of antitumor agents directly involves tumor growth, one well-known and useful approach is to use the severe combined immunodeficiency (SCID) mouse. The SCID mouse was discovered in 1983 and it has subsequently been shown to be defective in both T and B lymphocytes (7).

In this report, in an attempt to make chemotherapy more effective, we have examined the effect of coadministration of BLM and IND on tumor growth in normal and SCID mice.

## MATERIALS AND METHODS

### Materials

IND was obtained from Wako Pure Chemicals (Tokyo, Japan), BLM was purchased from Nihonkayaku (Tokyo, Japan), and all other chemicals used were of reagent grade.

### Mice

Female BALB/c and CB17 SCID mice were obtained from Clea (Tokyo, Japan) and were maintained under specific pathogen-free conditions. Body weight was measured twice a week between 9:00 and 11:00 a.m. All animal experiments were approved by the Institutional Animal Care and Use Committee and complied with the standards set out in the Guideline for the Care and Use of Laboratory Animals on the Takara-machi Campus of Kanazawa University.

### Cell Culture

Clone 20 (C20) was derived from the murine colon 26 adenocarcinoma cell line. These cell lines were grown as monolayer cultures in complete medium consisting of RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin G, and 100 µg/ml streptomycin at 37°C with 5% CO<sub>2</sub>.

### Inoculation of Tumors

The adherent cells were collected after a brief period of trypsinization and counted. Nine-week-old female mice were inoculated into the footpad of the right hind limb with  $1 \times 10^5$  tumor cells suspended in 0.04 ml sterilized endotoxin-free phosphate-buffered saline. The tumor size at the injected site was determined by measuring the dimensions of the footpad with calipers. On day 1 after tumor inoculation, treatment with 0.001% IND via the drinking water was commenced. On days 4, 6, and 8, BLM was administered intraperitoneally at a dose of 5 mg/kg. The tumor incidence (number of mice with a tumor/number of mice inoculated) was calculated after a period of 14 days and 21 days for animals treated without IND or with IND, respectively. When BALB/c mice rejected C20, they were inoculated with C20 into the left footpad, at  $1 \times 10^5$  tumor cells per mouse. After this second inoculation, mice were not treated with BLM or IND. The tumor incidence was calculated at 21 days after the second transplantation.

### In Vitro Sensitivity Assay of C20 Against BLM

Subconfluent cells were treated with trypsin and transferred to 24-well plates at a concentration of  $1 \times 10^5$  cells per well. After 24 h incubation, BLM was added to the culture media at the concentration indicated, with or without 10 µM IND. After a further 48 h incubation, these cells were treated with trypsin and then counted.

## RESULTS AND DISCUSSION

As shown in Fig. 1, tumor growth was partially inhibited by BLM after three administrations when the drug was ad-

<sup>1</sup> Department of Clinical Pharmaceutics, Kanazawa University, Kanazawa, Japan.

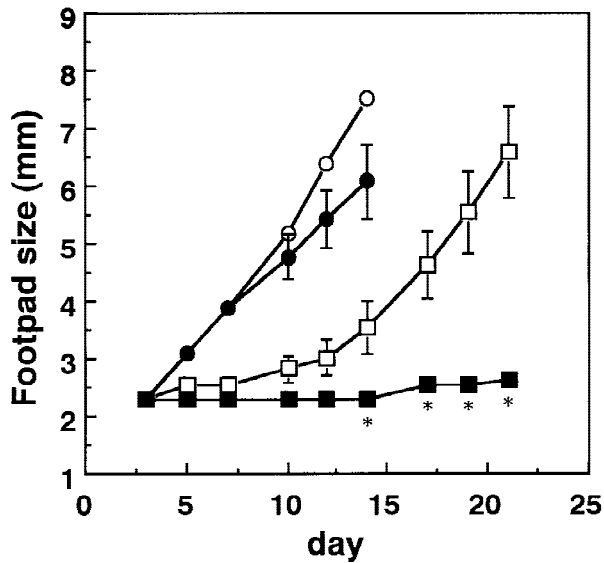
<sup>2</sup> Department of Pharmaceutics, Kyoritsu College of Pharmacy, Tokyo, Japan.

<sup>3</sup> Hospital Pharmacy, Kanazawa University, Kanazawa, Japan.

<sup>4</sup> Department of Pharmaceutics, Hokuriku University, Kanazawa, Japan.

<sup>5</sup> To whom correspondence should be addressed. (e-mail: nakashima-em@kyoritsu-ph.ac.jp)

**ABBREVIATIONS:** BLM, bleomycin; IND, indomethacin; PG, prostaglandin; SCID, severe combined immunodeficiency.



**Fig. 1.** Effects of BLM and IND on C20 local tumor growth. BALB/c mice were inoculated with C20 into the right footpad at  $1 \times 10^5$  cells/mouse, and injected i.p. with 5 mg/kg BLM on day 4, 6, 8, and given IND in drinking water (0.001%), beginning on the day after tumor inoculation. (○), C20 cells; (●), C20 cells with BLM; (□), C20 cells with IND; (■), C20 cells with BLM and IND. Each point and vertical bar represent the mean  $\pm$  SEM. \*Significantly different from the IND alone group ( $P < 0.05$ ).

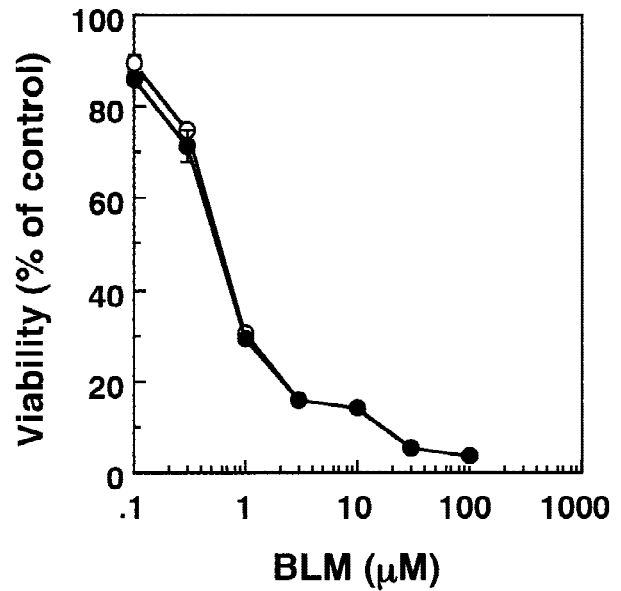
ministered on its own. IND was effective up to 2 weeks after tumor inoculation. However, after 2 weeks, the growth rate of the tumor was almost the same as that of the control. On the other hand, a marked synergistic effect was observed, such that the tumor growth was almost completely suppressed, when IND and BLM were coadministered.

We examined whether IND was able to increase the sensitivity of C20 to BLM. The plasma concentration of IND in mice, measured by HPLC, was about 3–6  $\mu$ M. Therefore, we cultured C20 cells in the indicated concentration of BLM, with or without 10  $\mu$ M IND. IND did not affect the dose-dependence of BLM toxicity on C20 (Fig. 2). These results suggested that the synergistic effect was not due to a direct action of IND on C20.

To investigate the mechanism governing the synergistic effect, we inoculated SCID mice with C20 tumor cells. BLM or IND had no effect on tumor growth after treatment with each drug on its own (Fig. 3). Moreover, little effect was observed when BLM and IND were administered together. Although it is not clear whether BLM and IND act directly on lymphocytes, these results indicate that lymphocytes are essential for the synergistic effect of BLM and IND.

To examine whether tumor immunity was induced in normal mice, we reinoculated C20 tumor cells into the opposite foot of the mice that had rejected the tumor. Seven out of 10 reinoculated mice rejected the tumor without BLM or IND. These data suggest that both BLM and IND act as activators of tumor immunity.

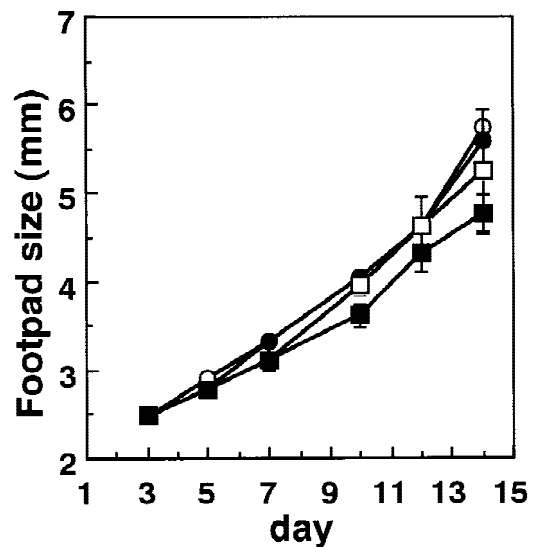
So far, the direct target for the BLM remains unclear, but it has been reported that BLM acts on KDH-8 hepatoma cells to reduce tumor growth factor-beta (TGF- $\beta$ ) secretion (6). It has also been reported that TGF- $\beta$  is secreted from other subclones of colon 26 cells *in vitro* (8), and that the concen-



**Fig. 2.** Effects of IND on in vitro sensitivity of C20. Cells were cultured with various concentrations of BLM for 48 h, with or without 10  $\mu$ M IND. (○), C20 cells with BLM; (●), C20 cells with BLM and IND. Each point indicates the mean  $\pm$  SEM.

tration of TGF- $\beta$  in serum is positively correlated with tumor size (9). It has been reported that TGF- $\beta$  reduces the number of interleukin-2 (IL-2) receptors on T cells as well as IL-2 dependent T cell growth (10). Our results indicate that lymphocytes are essential for the effect of the BLM because BLM is ineffective in SCID mice (Fig. 3). It may be that BLM acts on colon 26 C20 cells *via* a reduction in TGF- $\beta$ , similar to KDH-8 cells, and then T cell activation by IL-2 is increased.

There have been several reports showing that IND in-



**Fig. 3.** Effects of BLM and IND on C20 local tumor growth in CB-17 SCID mice. CB-17 SCID mice were inoculated with C20 into the right footpad at  $1 \times 10^5$  cells/mouse, and injected i.p. with 5 mg/kg BLM on day 4, 6, 8, and given IND in drinking water (0.001%), beginning on the day after tumor inoculation. (○), C20 cells; (●), C20 cells with BLM; (□), C20 cells with IND; (■), C20 cells with BLM and IND. Each point and vertical bar represent the mean  $\pm$  SEM.

hibits tumor growth and exhibits a synergistic effect with other biological response-modifiers (3,4). Its site of action, however, remains unclear. Ogino et al. have reported that IND reduces telomerase activity in tumor tissue and suppresses tumor growth in conventional and nude mice to the same extent (11). Our results indicate that IND has no effect on tumor growth in SCID mice (Fig. 3). Although these two investigations differed, as far as the route of administration was concerned, the doses were similar. So far, the reason for the difference remains unclear, but it may be that differences in subclones are responsible because there are many subclones of colon 26 (e.g., clone 5) which do not induce cachexia.

It has also been reported that IND suppresses cachexia and aids survival even in the advanced phase (2). IND is also effective in reducing cancer cachexia in other tumor models and in tumor patients (5,12,13) and, therefore, IND may well have a role to play in cancer chemotherapy.

In conclusion, IND and BLM exhibit a strong synergistic inhibitory effect on *in vivo* C20 tumor growth and both BLM and IND may act as activators of tumor immunity. These data indicate that the combination of BLM and IND will be useful in cancer chemotherapy.

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