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SHORT COMMUNICATIONS

Evidence that indomethacin reversibly inhibits cell growth in the G_1 phase of the cell cycle

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Our earlier studies have shown that indomethacin and aspirin in pharmacological doses inhibit the growth of a transplantable mast cell tumor (P815) and Lewis lung carcinoma in mice [1]. In subsequent studies, a variety of nonsteroidal anti-inflammatory drugs were found to inhibit growth and metabolic activity of transformed (rat hepatoma, HTC) and nontransformed (human fibroblast) cell lines in culture [2]. Their order of potency in inhibiting growth was identical to their reported anti-inflammatory activity. Metabolites and derivatives of the anti-inflammatory drugs that were pharmacologically inactive had little effect on culture growth. The drugs were not cytotoxic, since HTC growth resumed once drug was removed [2, 3]. In this communication, we present data to show that in HTC cultures upon removal of drug there is a 24-hr lag before resumption of growth, and that during this period there is a surge and decline in DNA synthesis. These and other data suggest that in the presence of anti-inflammatory drugs, the cells are arrested in the G_1 phase of the cycle and that the 24-hr delay represents the time required for the cells to traverse the remainder of the cell cycle.

HTC cells were grown in Eagle's medium supplemented with Earle's salts, 2 mM glutamine and 10% fetal calf serum in an atmosphere of 95% air and 5% CO_2 at 37°. For individual experiments, cells from confluent cultures were suspended by shaking the flasks and diluted 20-fold with fresh medium. One-ml aliquots (~50,000 cells) of the diluted suspension were dispensed into individual wells of Costar tissue culture cluster plates (16 mm wells, Cat. No. 3524, Cambridge, MA). The plates were incubated under the same conditions as the mother cultures.

Indomethacin was chosen for detailed study, since our earlier experiments had shown that it inhibited culture growth in lower concentrations (0.01 to 0.5 mM) than did most other anti-inflammatory drugs. The concentration of drug used, 0.4 mM, was one that completely suppressed culture growth [2].

Samples (100 μl) of a neutralized solution of indomethacin (4 mM) in Eagle's medium or medium without drugs were added to each well at the times indicated. To remove the drug, the culture medium was removed by aspiration, the cultures were washed once with medium, and 1 ml of fresh medium was added. All solutions were sterilized by filtration.

To count cells, the cultures were washed with Ca^{2+}- and Mg^{2+}-free Dulbecco's medium and then incubated with a 0.025% (w/v) trypsin solution. The detachment of cells was assessed by microscopic examination and, when complete, the reaction was stopped by the addition of soybean trypsin inhibitor (0.5%, w/v). The cells were counted in a Neubauer counting chamber. Cell viability was determined by the ability of the cells to exclude trypan blue (0.05% solution). For determination of mitotic index, cultures were grown in petri dishes with glass slides. Colchicine (2 μg/ml) was added upon removal of indomethacin from the cultures. The slides were removed at various time periods thereafter, fixed with an ethanol–acetic acid mixture (1:3), air-dried, and then treated with Geimsa stain. The percentage of cells in mitosis was determined by examination of 300 or more cells.

DNA synthesis was measured by adding 1 μCi deoxyribose-[5-^{3}H]thymidine (5 Ci/m-mol, 1 Ci/ml) (Amersham/Searle Corp., Arlington Heights, IL) to the cultures for 30 min. The amount of isotope incorporated into the trichloacetic acid precipitable fraction was measured as described previously [2].

Cell proliferation was inhibited whether indomethacin was added at the start (Fig. 1A) or during (Fig. 1B) culture growth. There appeared to be a delay before proliferation was retarded (Fig. 1B) and a delay before full growth resumed after removal of drug (Fig. 1, panels A and B, and Fig. 2A). Cell count began to increase about 24 hr after removal of drug (Fig. 2A), then continued to increase in an exponential fashion at a rate that was similar to that observed in control cultures (Fig. 1). Cell viability was not impaired by indomethacin, even after growth of the cultures had been inhibited for 5–6 days. For example, in one experiment 1.3 ± 0.1 per cent and 1.2 ± 0.3 per cent (mean ± S. E. M., n = 6) of the cells in control cultures were nonviable on days 7 and 8 of culture growth, compared to values of 3.6 ± 0.7 per cent and 2.2 ± 0.4 per cent in indomethacin-treated cultures.

In the presence of indomethacin, only a few of the cells (0.6 per cent) were in metaphase, and no increase in mitotic activity was observed until 22 hr after removal of drug. At all time points before 22 hr, the mitotic index was less than 2 per cent. Thereafter, mitotic activity increased sharply and reached a peak (~ 70 per cent) by 24–28 hr.

Before the increase in cell count and mitotic activity were apparent, there was a marked surge in the rate of DNA synthesis which reached a maximum at about 18 hr and then declined to a minimum at 22–24 hr (Fig. 2B). Similar data were obtained in two additional experiments. At the point of maximum DNA synthesis, the rate of isotope incorporation per cell in the indomethacin-treated cultures in all three experiments was 16–25 times higher than in the control culture. After 24 hr DNA synthesis increased gradually to produce a second, shallower peak at 32 hr. The synchrony achieved in the first cycle appeared to be largely lost by the second cycle.

To determine whether cells continued their cycle once DNA synthesis had occurred, indomethacin was added back to cultures either before the surge in DNA synthesis (7 hr after washing the cultures) or at the point of maximum DNA synthesis (18 hr after washing the cultures). Replication of the cells was prevented when drug was added before the increase in DNA synthesis but not when drug was added at the later time point (Table 1). In the latter case, culture growth was re-arrested once the cell population had doubled (Table 1).

The above data provide several indications that indomethacin had arrested the growth of cells at an early stage of the cell cycle. These data include: the observation that there was a surge in DNA synthesis before cell count or mitotic activity had increased following removal of drug; the ability to prevent growth by adding indomethacin back to the cultures before DNA synthesis had occurred; and the 24-hr delay before growth resumed. The time course of these events is consistent with earlier findings of Martin

Table 1. Growth of HTC cultures following the removal of indomethacin for a period of 7 or 18 hr*

Hr after wash	Cell counts per well			
	Experiment 1		Experiment 2	
	Indomethacin present	Indomethacin removed for 7 hr	Indomethacin present	Indomethacin removed for 18 hr
18			55,000 ± 1100	56,100 ± 7700
46	55,800 ± 2800	55,300 ± 1700	44,800 ± 3100	99,000 ± 10,300
70	45,000 ± 3800	51,000 ± 3800	44,200 ± 3700	95,000 ± 8900

* Indomethacin (final concentration 0.4 mM) was added on day 2 of culture growth. On day 5 (cell counts, 55,000), cultures were washed free of indomethacin and, 7 or 18 hr later, drug was re-added to the cultures. The table shows the number of cells in these cultures and in those in which indomethacin was present throughout the period of the experiment. Values are expressed as the mean ± S. E. M. for six cultures.

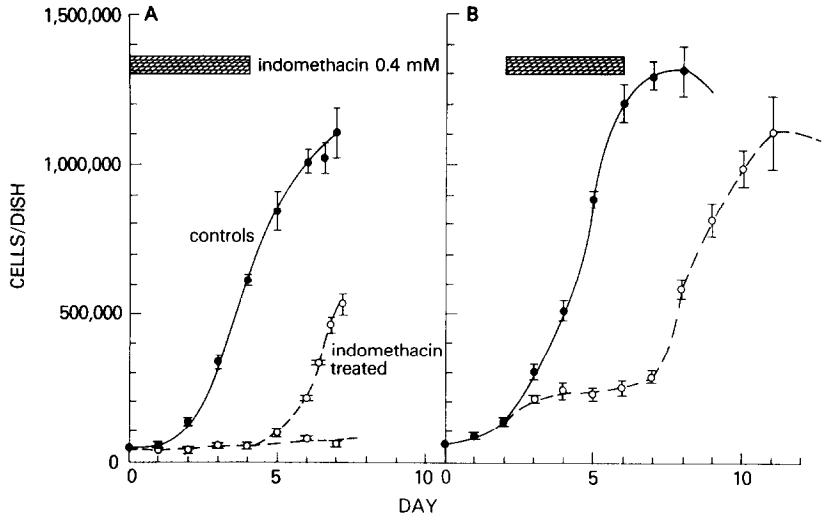

Fig. 1. Inhibition and resumption of HTC culture growth upon addition and removal of indomethacin. Indomethacin (0.4 mM final concentration) was added on day 0 (A) or 2 (B) of culture growth and was removed 4 days later as indicated by the hatched bar. In this and two additional experiments (data not shown), growth of cultures resumed after a delay of about 24 hr (open circles). The additional points at days 6 and 7 (panel A) depict the number of cells in cultures in which indomethacin was present throughout the entire 7-day period. Values are expressed as mean ± S. E. M. for six cultures.

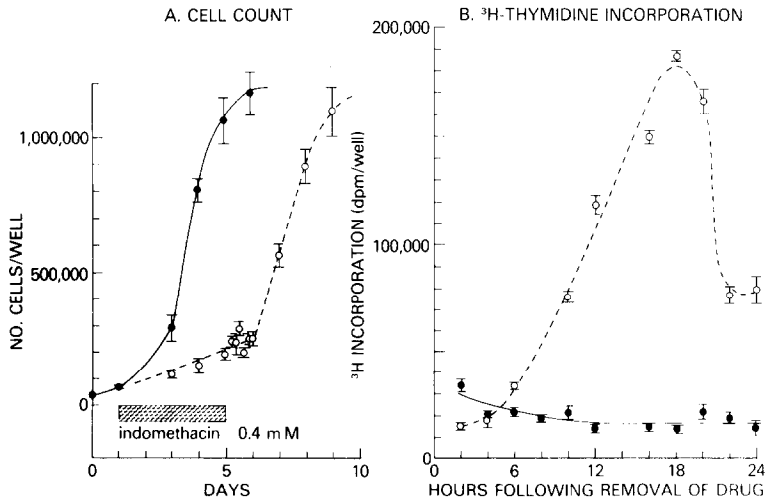

Fig. 2. HTC culture growth and DNA synthesis after removal of indomethacin. The drug (0.4 mM) was added on day 1 and removed on day 5. A period of 24 hr elapsed before cell counts increased (A), but during this period a large increase in DNA synthesis was apparent (B). Closed circles depict values for control cultures (no drug present) and open circles value for indomethacin-treated cultures. [^{3}H]-thymidine incorporation was determined for the 24-hr period between days 5 and 6 for both the indomethacin-treated and control cultures. Values are expressed as mean ± S. E. M. for six cultures.

et al. [4], who showed that in HTC cell culture maximum DNA synthesis occurred 17 hr after mitosis and that the full cycle required 24–25 hr for completion. These data suggest that growth had been arrested at the G_1 rather than the S or G_2 phase of the cycle. Other data support this conclusion. Cytophotometric measurement of the DNA content of individual cells in cultures of human fibroblasts showed that in the presence of 0.4 mM indomethacin more than 90 per cent of the cells are in the G_1 phase of the cycle compared to 38–40 per cent for control cultures in exponential growth (B. M. Bayer, H. Kruth, M. Vaughan and M. A. Beaven, unpublished data). Other workers using similar techniques have reported that, in the presence of 750 μM indomethacin, 96 per cent of HeLa cells acquire a DNA content that corresponds to the G_1 phase of the cycle [5].

The above data also suggest that, even after complete arrest of growth, viability of the cells is not impaired. More direct evidence for this has been obtained in autoradiographic studies of HTC cultures grown in the presence of labeled thymidine for varying periods of time. These studies indicated that more than 98 per cent of the cells had incorporated thymidine into nuclei 22 hr after removal of the indomethacin (B. M. Bayer, H. Kruth, M. Vaughan and M. A. Beaven, unpublished data).

The mechanism by which the anti-inflammatory drugs inhibit cell growth is unknown. The drugs do not appear to have a direct inhibitory action on protein or nucleic acid synthesis [2] or amino acid transport (unpublished data). Although there is a parallelism in the ability of the drugs to inhibit culture growth and prostaglandin synthesis [2], the antiproliferative action of the drugs is not reversed by the addition of prostaglandins of the A, B, E and F series [3].

Other workers have noted that replication of HeLa cells [5], rat fibroblasts [6] and rat lymphocytes [7], like that of HTC cells and human fibroblasts [2], is inhibited by the non-steroid anti-inflammatory drugs. The ability of salicylates [8–10] and other anti-inflammatory drugs [11, 12] to inhibit lymphocyte blast transformation induced by phytohaemagglutinin [8–10], antigen [8], or allogenic lymphocytes [10] may be a related phenomenon. This action is also reversible [9], and the drugs appear to affect the early stages of transformtion. In addition to their ability to inhibit prostaglandin synthesis [13], the ability of anti-inflammatory drugs to inhibit proliferation and lymphocyte transformation could contribute to their therapeutic action.

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Failure of propranolol to alter thyroid radioiodine uptake and serum concentrations of thyroxine and triiodothyronine in rats

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The beta-adrenergic blocking agent, propranolol, has been used for several years in the treatment of hyperthyroidism, e.g. with antithyroid drugs, before ^{131}I therapy has taken effect, in thyroid storm and in the preoperative preparation for thyroïdectomy (reviewed in Ref. 1). It has been used as the only drug before thyroidectomy [2] and even as the sole therapy [3]. Until recently, propranolol was thought to control peripheral manifestations of the disease without influencing thyroid function. Thus, it had no effect on thyroid radioiodine uptake [3–5], PBI [3, 6], PB[^{125}I] [5] or thyroid iodine release and peripheral thyroxine (T_4) turnover [7]. However, a number of recent reports [8–12] indicate that in hyperthyroid, euthyroid and hypothyroid T_4 maintained subjects propranolol treatment lowered serum triiodothyronine (T_3) concentrations. In some reports, serum T_4 was elevated [9, 11–13], while in others it was unchanged [8, 10] after propranolol administration. In three of four hyperthyroid patients treated with propranolol serum, PB[^{125}I] was elevated on day 8 after ^{125}I administration [4].

Three studies have been performed in rats [14–16]. In one study, propranolol induced striking increases in serum T_4 in both intact and hypophysectomized rats [14], while in the other two studies [15, 16] it had no effect. Propranolol did not influence the peripheral metabolism of T_4 and essentially did not affect thyroid ^{131}I uptake or the intrathyroid distribution of ^{131}I in iodoprotein [15]. Serum T_3 was not measured in any of these studies. The present report describes the failure of large doses of propranolol to influence serum T_4 and T_3 and thyroid ^{131}I uptake in rats.

Male Sprague–Dawley rats (Canadian Breeding Farms, St. Constant, Quebec), initially weighing 120–130 g, were divided into five groups, as indicated in Table 1. Group 1 was fed powdered Purina laboratory chow alone. Groups