Cytotoxicity of Clinically-Used Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) in the Human Bladder Cancer Cell Line UM-UC-3

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We evaluated 8 clinically-used non-steroidal anti-inflammatory drugs (NSAIDs): Aspirin, Etodolac, Diclofenac, Ibuprofen, Indomethacin, Mefenamic acid, Nabumetone, and Piroxicam, for cytotoxicity in the human bladder cancer cell line UM-UC-3 *in vitro*. Basing our dosages on the maximum concentration of each NSAID used clinically, four concentrations of each drug were prepared, and the viability of UM-UC-3 cells given each treatment was monitored for up to 5 days. At the highest concentration, Aspirin and Diclofenac caused no decrease in UM-UC-3 viability during the 5-day incubation. Etodolac, Ibuprofen, Indomethacin, and Piroxicam decreased the viability of UM-UC-3 cells about 20% on the fifth day. Treatment with 30 μg/ml of Mefenamic acid and 16 μg/ml of Nabumetone caused about a 40% loss of cell viability on the last day of the experiment. Thus, the most effective cytotoxicity was observed with Mefenamic acid and Nabumetone. We then examined the nature of the cell death induced by these two drugs, by biochemical and morphological analyses. A DNA fragmentation assay showed DNA-ladder formation with both Mefenamic acid and Nabumetone treatment. In addition, nuclear blebbing was observed by fluorescence microscopy in both Mefenamic acid- and Nabumetone-treated cells. Since DNA ladder formation and nuclear blebbing are hallmarks of typical apoptotic cell death, we concluded that at least two NSAIDs, Mefenamic acid and Nabumetone, could cause apoptotic cell death in the human bladder cell line, UM-UC-3.

Key words —— UM-UC-3, mefenamic acid, nabumetone, bladder cancer, apoptosis

INTRODUCTION

Since the discovery of the anti-inflammatory effect of salicylic acid in the late 19th century, anti-inflammatory drugs have been among the most common medicines, not only in hospitals but for domestic self-medication, as well. Today, non-steroidal anti-inflammatory drugs (NSAIDs) are well-recognized alternatives to steroidal drugs that share their anti-inflammatory, antipyretic, and analgesic pharmacological effects. Among these classically well-known pharmacological effects, the analgesic effect of NSAIDs is especially applied in the clinical field of cancer therapy.[1)]

For cancer patients, the struggle against cancer pain causes serious anguish in their daily life. To raise the quality of daily life (QOL) of cancer patients, some pain killers are routinely administered with anticancer drugs. Among these, morphine is the most popular and still the strongest pain-killer used for cancer patients. However, unfortunately, sometimes it becomes very hard for physicians to relieve sufficiently the pain of cancer patients with morphine alone.[2)] In some cases, physicians have found that the simultaneous administration of NSAIDs with morphine is a more effective treatment for releasing cancer patients from intolerable pain.[3)]

NSAIDs are recognized as the most fundamental analgesics next to morphine. An important problem associated with using morphine to treat cancer pain is that greater dosages are usually needed as the pain continues. Therefore, the World Health Organization (WHO) recommends the aggressive use of NSAIDs to relieve pain during cancer treatment.[1)] Pain treatment combining morphine and NSAIDs is also sometimes effective, as described above. As a consequence, the cancer cells in the patients' body are routinely exposed to NSAIDs from the time the

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cancer chemotherapy begins. These facts led us to wonder if the NSAIDs administered to cancer patients work only as pain-killers.[4] In other words, we have little knowledge of how NSAIDs might affect the physiology of cancer cells. If NSAIDs have some additional pharmacological effect in cancer cells as a cell-death inducers, it would be useful to consider this knowledge when determining new cancer chemotherapies.

In this study, we focused on human bladder cancer and used UM-UC-3 cells, a human-derived cultured bladder cancer cell line. We chose 8 representative NSAIDs that are very commonly used, and examined their effect on UM-UC-3 cell viability. Our findings indicated that two of the tested NSAIDs were relatively strongly cytotoxic to these cells, so we then investigated the nature of the cell death elicited by these drugs.

MATERIALS AND METHODS

Cell Line, Cell Culture, and Microscopy —— Human cultured bladder cell line UM-UC-3 cells[5] were purchased from Dai-Nippon Pharmaceutical Company (Osaka, Japan) and maintained in Dulbecco's Modified Eagle Medium (Nissui; Tokyo, Japan) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 1% non-essential amino acids for MEM Eagle Medium (ICN; Ohio, U.S.A.), 100 U/ml penicillin, and 100 μg/ml streptomycin. The UM-UC-3 cells were grown under 100% humidity in a 5% CO_2 atmosphere at 37°C. The cell morphology was observed using an Olympus model IX-70 inverted phase-contrast microscope equipped with an IX-FLA fluorescence observation device.

Administration of NSAIDs —— Eight NSAIDs (Aspirin, Asp; Diclofenac, Dic; Etodolac, Eto; Ibuprofen, Ibu; Indomethacin, Indo; Mefenamic acid, Mef; Nabumetone, Nab; and Piroxicam, Pir, all purchased from SIGMA, MO, U.S.A.) were dissolved in 100% ethanol, and 4 different concentrations of each NSAID were tested for their effect on UM-UC-3 cell viability. The concentrations used for the viability experiment were: Asp, 500 μg/ml, 100 μg/ml, 50 μg/ml, and 1 μg/ml; Dic, 1 μg/ml, 500 ng/ml, 100 ng/ml, and 10 ng/ml; Eto, 8 μg/ml, 2 μg/ml, 500 ng/ml, and 100 ng/ml; Ibu, 12 μg/ml, 6 μg/ml, 1 μg/ml, and 500 ng/ml; Indo, 4 μg/ml, 2 μg/ml, 500 ng/ml, and 100 ng/ml; Mef, 30 μg/ml, 10 μg/ml, 1 μg/ml, and 500 ng/ml; Nab, 16 μg/ml, 8 μg/ml, 1 μg/ml, and 500 ng/ml; Pir, 400 ng/ml, 200 ng/ml, 50 ng/ml, and 10 ng/ml. As a control, cells were treated with the solvent (100% ethanol), alone. UM-UC-3 cells were routinely cultured to *ca*. 70% confluence in medium alone, then half a day before the drug administration, the culture medium was changed. The cells were kept in the CO_2 incubator until they were harvested.

Cell Viability —— Cell viability was assessed using the AlamarBlue™ assay (Tresk Diagnostic Systems, OH, U.S.A.) according to the manufacturer's instructions. This assay uses a fluorometric growth indicator that responds to metabolic activity. Specifically, it uses an oxidation-reduction indicator that fluoresces and changes color in response to chemical reduction of the culture medium resulting from cell growth. In brief, about 10^4 cells/100 μl medium were plated in a 96-well micro-titer plate, and the NSAIDs were added. Next, 10 μl of AlamarBlue™ was added and the cells were kept in a CO_2 incubator for 4 hr. The colorimetric assay was conducted by measuring the absorbance at 570 and 600 nm and the viability was calculated using the following equation.

$$Viability(\%) = 100 \times \frac{SV - bV}{cV - bV}$$

where,

SV: the subtracted value of Abs at 570 nm from Abs at 600 nm of the sample well,

bV: the subtracted value of Abs at 570 nm from Abs at 600 nm of the blank well,

cV: the subtracted value of Abs at 570 nm from Abs at 600 nm of the control well.

The "blank" means the medium and the "control" means the culture without NSAIDs.

Morphological Observation of Apoptosis, and DNA Fragmentation Assay —— Typical apoptotic nuclear-DNA condensation or blebbing was used as the morphological marker of apoptosis, and was assessed by staining the nuclear DNA with Hoechst 33258 dye (Molecular Probes, U.S.A.), as described previously.[6,7] DNA-ladder formation was documented by electrophoresis on a 1.8% agarose gel, as reported previously.[8] In brief, harvested cells were washed with phosphate-buffered saline (PBS) without $CaCl_2$ and $MgSO_4$ [PBS(–)] and suspended in cell lysis solution (0.1% Triton X-100, 50 mM Tris–HCl [pH 7.5], 5 mM EDTA) for 30 min. The supernatant of the cell lysate was collected by centrifugation at $10000 \times g$ at 4°C and was followed by RNase (Boehringer Mannheim, Germany) digestion (200 μg/ml) at 37°C for 2 hr. Digestion with

200 μg/ml proteinase K (Boehringer Mannheim) at 37°C overnight was then performed. Cellular DNA was ethanol precipitated, separated by electrophoresis on a 1.8% agarose gel, and stained with ethidium bromide.

RESULTS AND DISCUSSION

We investigated the viability of UM-UC-3 cells for five days after drug administration (Day 0 to Day 4). During the five-day viability experiment, the culture medium was not changed. The cell viability was assessed each day for five days using the AlamarBlue™ assay (Fig. 1). As described in the Materials and Methods section, we tested four concentrations of each NSAID. The highest drug concentration was calculated from the clinical dosage (maximum dose per day/70 kg [average body weight of an adult male] times 2/3 l [common ratio of total body fluid *vs*. body weight]) of each NSAID. However, in the case of Asp, the highest dosage as calculated above was extremely acidic and caused some precipitation in the culture medium. Therefore, for this drug we sequentially decreased the concentration and determined a new maximum dosage for the experiment as the highest concentration that did not cause any visible changes to the culture medium.

From the viability curves, we could divide the 8 NSAIDs into three groups: 1) drugs that caused almost no change in the UM-UC-3 viability during the 5-day experimental term; this group included Asp and Dic, 2) drugs that caused a maximum of 20% loss of viability on the last day of the viability experiment using the highest dosage; this group included Eto, Ibu, Indo, and Pir, and 3) drugs that caused a loss of viability of about 30–35% on the last day at the highest dosage; this group included Mef and Nab. In parallel to the AlamarBlue™ viability assay, we observed the UM-UC-3 cell morphology by microscopic observation. Usually, when adherent cells start dying for any reason, the cells become round and start floating in the medium. However, interestingly, we observed few floating cells in group 2) and almost all of the cells were still adherent on the last day of the viability experiment (data not shown).

Since the decrease in viability on the last day of the viability experiment was greatest in the cases of Mef and Nab administration, we focused on the nature of the cell death caused by these two drugs, especially whether or not it was apoptotic. This was because recent studies report that NSAIDs sometimes cause apoptotic cell death, not only in cultured cancer cell lines, but also in experimental animals with cancer.[9,10] Therefore, on the last day of the viability experiment we harvested the Mef- and Nab-treated UM-UC-3 cells that had received the highest dose of each drug, and evaluated the nature of the cell death in each case.

There are various strategies for evaluating apoptotic cell death. Here, we used two well-known biochemical and morphological methods for assessing apoptosis. The biochemical test is a DNA fragmentation assay.[8] The final step of apoptotic cell death is defined as DNA cleavage, because the cleaved DNA is no longer a "genetic construction map" and cannot support cell survival. Some apoptosis-specific DNases[11–13] are thought to digest DNA every 180 bp, at points that are exposed due to the structure of chromatin. As a consequence of this digestion pattern, a "DNA ladder" appears on agarose gels following electrophoresis.

Figure 2A shows the results of the DNA fragmentation assay. Intact (lane "I" next to the leftmost lane) UM-UC-3 cells showed no DNA ladder formation nor did the solvent-only control cells ("S" lane) on the 4th day. On the other hand, on the 4th day after drug administration, Mef-treated UM-UC-3 cells showed a smeary DNA ladder, and the Nab-treated cells showed a clear DNA ladder. The intensity of the DNA ladder was lower in the Mef-treated than the Nab-treated UM-UC-3 cells. Because the concentration of Mef used was greater than that of Nab (Mef; 30 μg/ml, Nab; 16 μg/ml), the reason for the difference in DNA-ladder intensity is unclear. Furthermore, Nabumetone is a prodrug, and the pharmacologically active form is its metabolite, 6-methoxy-2-nafutyl-aceticacid (6MNA).[14] One plausible explanation for the difference in DNA-ladder intensity is that, because several DNases play roles in apoptosis, different NSAIDs may stimulate the activation of different DNases.

Historically, apoptosis was identified by characteristic morphological features of cells. The most distinct morphological characterization of apoptosis is "nuclear DNA condensation and blebbing."[15] It is well known that, at relatively late stages of apoptosis, nuclear DNA condenses and causes nuclear blebbing. This morphological feature is easily detected by staining the nuclear DNA with a DNA-binding fluorescent dye, such as Hoechst 33258. Thus, we harvested the UM-UC-3 cells on the 4th day after drug administration, stained them

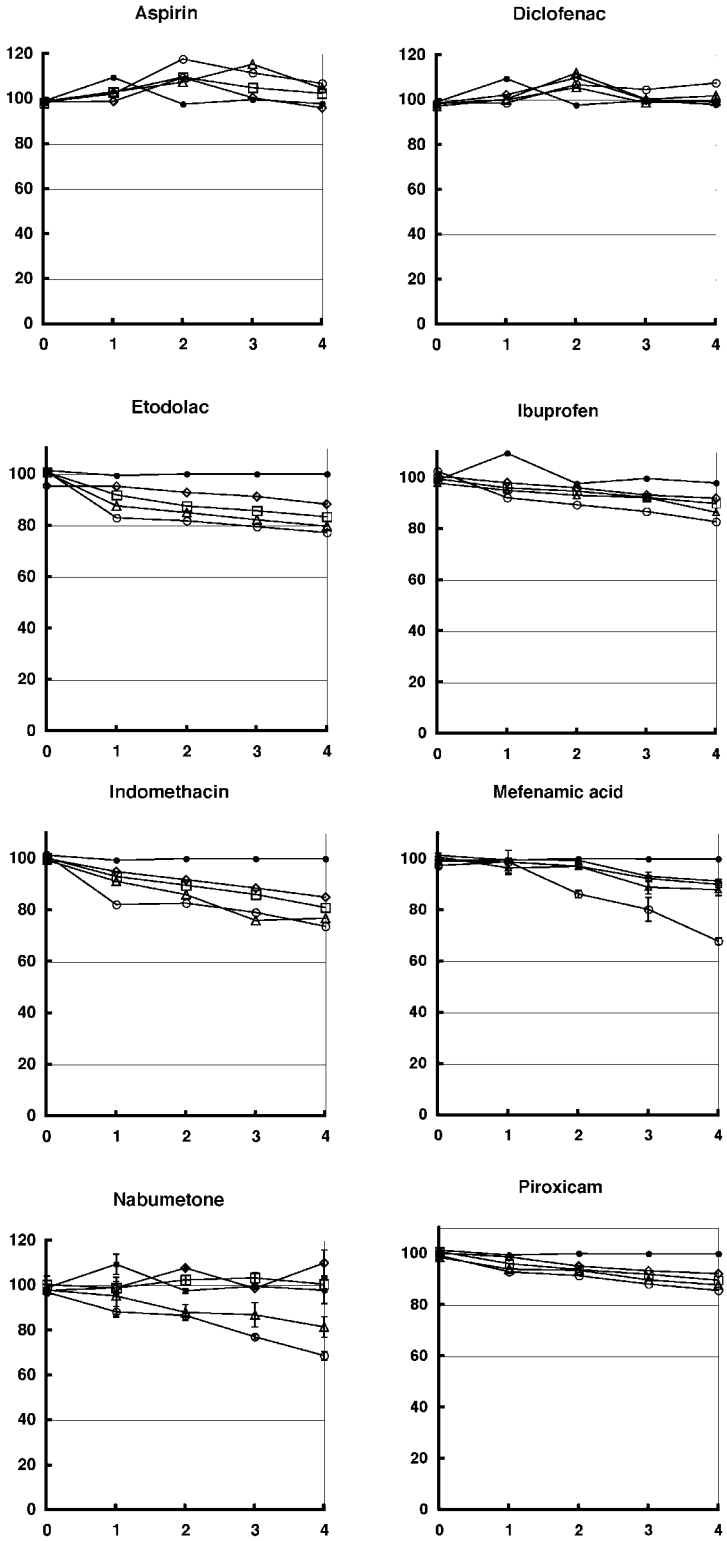

Fig. 1. Viability of UM-UC-3 Cells after Administration of NSAIDs

The viability of UM-UC-3 cells was examined for 5 days after treatment with 8 different NSAIDs, listed above each graph. The symbols used for the viability experiment are: Asp, circle (500 μg/ml), triangle (100 μg/ml), squire (50 μg/ml), and diamond (1 μg/ml); Dic, circle (1 μg/ml), triangle (500 ng/ml), squire (100 ng/ml), and diamond (10 ng/ml); Eto, circle (8 μg/ml), triangle (2 μg/ml), squire (500 ng/ml), and diamond (100 ng/ml); Ibu, circle (12 μg/ml), triangle (6 μg/ml), squire (1 μg/ml), and diamond (500 ng/ml); Indo, circle (4 μg/ml), triangle (2 μg/ml), squire (500 ng/ml), and diamond (100 ng/ml); Mef, circle (30 μg/ml), triangle (10 μg/ml), squire (1 μg/ml), and diamond (500 ng/ml); Nab, circle (16 μg/ml), triangle (8 μg/ml), squire (1 μg/ml), and diamond (500 ng/ml); Pir, circle (400 ng/ml), triangle (200 ng/ml), squire (50 ng/ml), and diamond (10 ng/ml). The closed circle was indicated as a solvent control in each graph. In case of Mef and Nab, cell viability is expressed as the mean ± S.E. of the values obtained from three independent experiments. The numbers on X-axis on each graph indicates the days after NSAIDs administration and numbers on Y-axis on each graph indicates the viability (%) of UM-UC-3 cells.

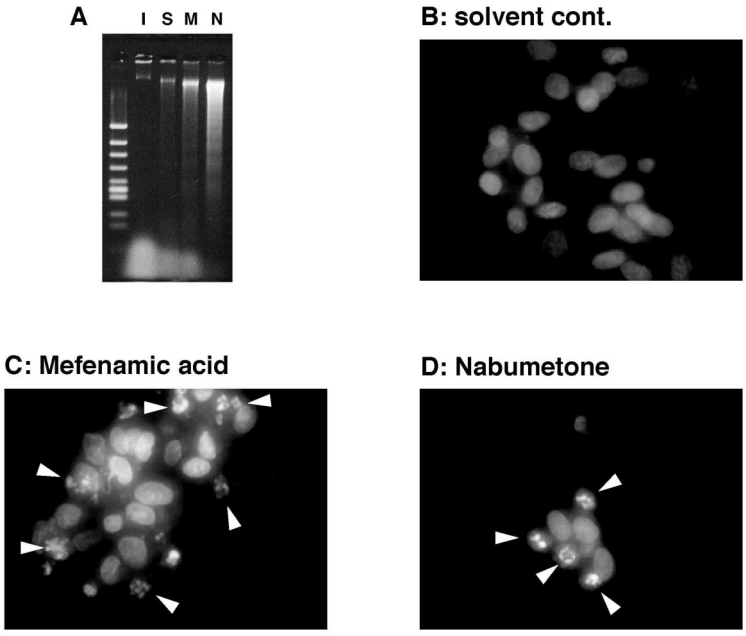

Fig. 2. Biochemical and Morphological Evidence of Apoptotic Cell Death in UM-UC-3 Cells Caused by Mef and Nab

A: DNA fragmentation assay. DNA was extracted and separated by electrophoresis on 1.8% agarose gels (see Materials and Methods). The leftmost lane shows ϕX174/*Hinc* II-digested molecular weight markers. The DNA sample in the second from the leftmost lane was from intact UM-UC-3 cells (I). "S," "M," and "N" above the remaining lanes indicate samples from the solvent control, and Mef-, and Nab-treated samples, respectively. Sample "I" was collected on the starting day of the experiment. Samples "S," "M," and "N" were collected on the 4th day after drug or solvent-only administration. This figure shows representative data from three independent experiments. B; C; D: Typical phase-contrast micrograph images of UM-UC-3 cells following treatment with: B, 100% ethanol, the solvent; C, Mef (30 μg/ml); and D, Nab (16 μg/ml). UM-UC-3 cells were examined for nuclear blebbing after administration of the drugs indicated at the top of each panel. The white arrowheads indicate apoptotic cells showing typical nuclear blebbing. Magnification = original $\times$ 200. These data are representative of three independent experiments.

with Hoechst 33258, and observed them by fluorescence microscopy. The results are shown in Figs. 2B–2D.

The solvent control showed absolutely no nuclear-DNA condensation or blebbing in any cell under fluorescence microscopy (Fig. 2B). In contrast, the Mef- and Nab-treated UM-UC-3 cells showed distinct nuclear-DNA condensation, as indicated by white arrowheads in Figs. 2C and 2D. Moreover, the proportion of cells showing nuclear-DNA fragmentation in the microscopic field coincided closely with the viability data on the last day of the viability experiment (data not shown). Therefore, taking the results of the DNA fragmentation assay and fluorescence microscopic observation together, we concluded that Mef and Nab can induce apoptotic cell death in the UM-UC-3 human bladder cancer cell line.

Although the pharmacological effect of Mef and Nab is the inhibition of cyclooxygenase (COX)-1, and -2,[14,16] the results of this study present the interesting observation that Mef and Nab also act as apoptosis-inducing drugs in the cultured cancer cell line, UM-UC-3. However, it is too early to apply the results of this study to clinical cancer chemotherapy. The physiological environment around cancer cells is very different between *in vivo* and *in vitro* situations. It is likely that some unknown physiological factors would affect the pharmacological activity of Mef and Nab as apoptosis inducers in bladder cancer patients. Despite these issues, further intensive studies investigating the detailed mechanisms of these death-signal transduction pathways could help us better understand these findings and ultimately lead to their clinical applications.

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