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.4)

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...
cancer chemotherapy begins. These facts led us to wonder if the NSAIDs administered to cancer patients work only as pain-killers. 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 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% CO2 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. Our materials and methods section is concluded by noting 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.
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™ test, we harvested the 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. 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 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). 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.” 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), square (50 µg/ml), and diamond (1 µg/ml); Dic, circle (1 µg/ml), triangle (500 ng/ml), and square (100 ng/ml); Eto, circle (8 µg/ml), triangle (2 µg/ml), square (500 ng/ml), and diamond (100 ng/ml); Ibu, circle (12 µg/ml), triangle (6 µg/ml), square (1 µg/ml), and diamond (500 ng/ml); Indo, circle (4 µg/ml), triangle (2 µg/ml), square (500 ng/ml), and diamond (100 ng/ml); Mef, circle (30 µg/ml), triangle (10 µg/ml), square (1 µg/ml), and diamond (500 ng/ml); Nab, circle (16 µg/ml), triangle (8 µg/ml), square (1 µg/ml), and diamond (500 ng/ml); Pir, circle (400 ng/ml), triangle (200 ng/ml), square (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.
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.

Acknowledgements This work was supported by a Nihon University General Individual Research Grant for 2003 (to F. K.) and a Grant from the Ministry of Education, Culture, Sport, Science, and Technology of Japan to promote multi-disciplinary research (to F. K. and M. A.).

REFERENCES


