Direct toxicity of nonsteroidal antiinflammatory drugs for renal medullary cells

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Antipyretic analgesics, taken in large doses over a prolonged period, cause a specific form of kidney disease, characterized by papillary necrosis and interstitial scarring. Epidemiological evidence incriminated mixtures of drugs including aspirin (ASA), phenacetin, and caffeine. The mechanism of toxicity is unclear. We tested the effects of ASA, acetaminophen (APAF, the active metabolite of phenacetin), caffeine, and other related drugs individually and in combination on mouse inner medullary collecting duct cells (mIMCD3). The number of rapidly proliferating cells was reduced by ≈50% by 0.5 mM ASA, salicylic acid, or APAF. The drugs had less effect on confluent cells, which proliferate slowly. Thus, the slow *in vivo* turnover of IMCD cells could explain why clinical toxicity requires very high doses of these drugs over a very long period. Caffeine greatly potentiated the effect of acetaminophen, pointing to a potential danger of the mixture. Cyclooxygenase (COX) inhibitors, indomethacin and NS-398, did not reduce cell number except at concentrations greatly in excess of those that inhibit COX. Therefore, COX inhibition alone is not toxic. APAF arrests most cells in late G_1 and S and produces a mixed form of cell death with both oncosis (swollen cells and nuclei) and apoptosis. APAF is known to inhibit the synthesis of DNA and cause chromosomal aberrations due to inhibition of ribonucleotide reductase. Such effects of APAF might account for renal medullary cell death *in vivo* and development of uroepithelial tumors from surviving cells that have chromosomal aberrations.

acetaminophen | aspirin | salicylic acid | indomethacin | caffeine

Combinations of nonsteroidal antiinflammatory drugs (NSAIDs), taken in large doses over a prolonged period, cause a specific form of kidney disease, characterized by papillary necrosis and interstitial scarring (1). The patients have progressive chronic renal failure and are susceptible to the subsequent development of uroepithelial tumors. It is not clear what the mechanism of the drug toxicity is, but there are many theories, including the effects of cyclooxygenase (COX) inhibition, direct toxicity owing to elevated concentration of the drugs in the medulla, anoxia, and metabolic effects (2, 3). Epidemiological evidence incriminated mixtures of drugs including aspirin (ASA), and phenacetin or acetaminophen (APAF), the active metabolite of phenacetin (4). The mixtures often also contained caffeine. Interestingly, in some epidemiological studies caffeine content was the best predictor for subsequent renal failure (5, 6). The renal medullary toxicity of these particular analgesic drugs was apparently supported by animal studies, but interpretation of both the epidemiological and animal studies has been questioned (7–9).

There is also considerable evidence that NSAIDs slow the growth of tumor cells and cause them to die by apoptosis (10, 11). Those actions involve the inhibition of COX in some cases, but not in others. Furthermore, caffeine influences many pathways involved in the cellular response to DNA damage, reducing the cell cycle delay caused by DNA damage and inhibiting repair of the damage (12).

We hypothesized (*i*) that the NSAIDs and caffeine, which were implicated in renal medullary necrosis, may be directly toxic to renal inner medullary cells, causing apoptosis; (*ii*) that the toxicity could be enhanced by combinations of the drugs; and (*iii*) that the toxicity might be related to the inhibition of COX. To test these hypotheses we exposed renal inner medullary collecting duct (mIMCD3) cells that were rapidly proliferating or confluent to salicylic acid (SA), ASA, APAF, NS398, indomethacin, and caffeine, either individually or in combinations, and examined the effects on cell number, cell cycle, and cell death. We find evidence of direct toxicity of all of these drugs. Toxicity is particularly evident with the combination of caffeine and APAF. We also found that COX inhibition *per se* is not sufficient to produce these effects and that APAF kills the cells by oncosis as well as apoptosis. [The term *oncosis* (derived from *onkos*, meaning swelling) was proposed in 1910 by Von Recklinghausen precisely to mean cell death with swelling. Oncosis leads to necrosis with karyolysis and stands in contrast to apoptosis, which leads to necrosis with karyorhexis and cell shrinkage (13, 14).] Furthermore, toxicity is considerably greater in rapidly proliferating cells than in more slowly growing, confluent cells.

PHARMACOLOGY

Materials and Methods

Cell Culture. mIMCD-3 cells (15), generously provided by S. Gullans (Harvard Medical School), were used in passages 14 to 20. They were grown in medium containing 45% DMEM (low glucose), 45% Conn's improved medium mF12 (Irvine Scientific), 10% FBS, and L-glutamine (2 mM) (Life Technologies, Rockville, MD) at 37°C with 5% CO_2 plus 95% air. The osmolality of this medium was 300 mosm/kg (freezing-point osmometer; Advanced Instruments, Norwood, MA). Cells were studied either in the exponential phase of growth (rapidly proliferating cells) in the presence of FBS or postconfluence (confluent cells), either with or without FBS.

Drugs. APAF (4-acetoaminophenol, lot 107H0332), acetylSA (lot 46H105325), caffeine (lot 77H01221), SA (lot 48H0206), and indomethacin (lot 38H0906) were from Sigma. NS398 (lot 12923p) was from Cayman (Ann Arbor, MI). All drugs were directly dissolved in the medium except indomethacin and NS398, which were dissolved in DMSO and then diluted with medium to a final DMSO concentration of 0.1% or less. The same concentration of DMSO was added to control cells, as appropriate.

Abbreviations: NSAIDs, nonsteroidal antiinflammatory drugs; APAF, acetaminophen; SA, salicylic acid; ASA, aspirin; LSC, laser scanning cytometer; COX, cyclooxygenase; PI, propidium iodide; mIMCD3 cells, mouse inner medullary collecting duct cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PGE_2, prostaglandin E_2.

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Experimental Design. To study cells in the log phase of growth (rapidly proliferating cells), 1,000 cells were plated for 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay, or 2,000 cells were plated for laser scanning cytometry (LSC) assay, then after 8 h for attachment, the control medium was replaced by an otherwise identical one, containing one or more drugs. The media were renewed 2 days after the drugs were first added, and the cells were analyzed after 3–4 days. Experiments were performed in the presence of 10% FBS, except for some experiments in confluent condition.

The MTT assay was used to estimate cell number as previously described (16, 17). Briefly, mIMCD3 cells were seeded on 96-well plates (Costar 3596) and were allowed 8 h to attach. Then, control or experimental media were substituted and remained for the time required by the experiment. At the termination of the experiment all media were replaced with serum-free control medium, containing MTT, 1 mg/ml, for 2 h at 37°C. After the MTT solution was aspirated, the blue formazan reaction product was extracted from the cells with propanol for 10 min with shaking, then the optical density was read on a Labsystems Multiskan MCC/340 microplate scanning spectrophotometer at 540 nm with background subtraction at 690 nm. The result for each condition in a single experiment was determined as the mean of measurements of eight separate wells.

Flow cytometry was used to analyze cell cycle and apoptosis, as previously described (17). In brief, mIMCD3 cells were seeded (10^5 cells per dish) in 10-cm dishes in 300 mosmol/kg medium. After 8 h, prewarmed control medium or otherwise identical medium containing the drugs was substituted for 3 to 4 days. After the end of the experiment, the floating cells and particles suspended in the media were harvested by centrifugation at 500 $180 \times g$ for 5 min, and the pellet was suspended in PBS. The attached cells were harvested with trypsin and combined with the floating cells. The mixed cells and particles were centrifuged at $500 \times g$ for 5 min, resuspended in 500 μl of PBS, then fixed by adding them to ice-cold 70% ethanol, and stored at 20°C. The fixed cells were washed once with PBS, then stained with propidium iodide (PI) (80 μg/ml) in the presence of 1 mg/ml RNase A in PBS for 30 min at room temperature in the dark. A Coulter flow cytometer (model EPICS-XL-MCL) was used. Cell cycle compartments were deconvoluted from a single-parameter DNA histogram of 30,000 events with the use of WINCYCLE (Phoenix Flow Systems, San Diego, CA) after exclusion of cell doublets (Win MDI 2.7, Joseph Trotter, The Salk Institute for Biological Studies), and results were expressed as a percentage of cell population.

LSC. Cells on eight-chamber plastic slides (Nalgene Nunc International) were fixed in 100% methanol at −20°C for 15 min, incubated with 0.1% Triton X-100 for 4 min, stained with 10 μg/ml PI plus 1 mg/ml RNase (Sigma) for 15 min at room temperature, then mounted with 200 μl of antifade (Molecular Probes). A laser scanning cytometer (Compucyte Corporation, Cambridge, MA) with a ×20 objective (Olympus BX50) was used to count the number of cells (nuclei) and to quantify the PI staining in each nucleus. The integral of PI fluorescence in each nucleus (total PI fluorescence), which is proportional to DNA content, was used to determine position in the cell cycle (G_1, S or G_2/M). The data are displayed as cytograms, with the number of cells plotted versus DNA content

Transmission Electron Microscopy. Cells were scraped, resuspended in control medium, centrifuged ($800 \times g$ for 5 min), then fixed in 1.25% glutaraldehyde in cacodylate buffer containing 1% $CaCl_2$ at 4°C. The fixed cells were washed three times in Sabatini's solution (PBS with 6.8% sucrose), then the pellets were cut into small (1 mm^3) cubes, postfixed with 1% osmium tetroxide for 1 h, washed three times in Sabatini's solution, and dehydrated by passage through graded concentrations of ethylene alcohol, followed by sequential treatment with propylene oxide, a 1:1 Epon-propylene oxide mix, and three changes of pure Epon, as previously described (18). Polymerization was at 60°C overnight. Ultrathin sections were cut with a Leica Ultracut UCT Ultra Microtome with a Dupont diamond knife, stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (JEOL 1200EXII) at an accelerating voltage of 80 kV, and a 40-mm objective aperture.

COX Activity. Cells were incubated in control medium or media containing drugs for 4 days. Arachidonic acid (5 μM) (lot 12284h; Cayman) added during the last 24 h. Prostaglandin E_2 (PGE_2) in the medium was measured by immunoassay (Cayman), according to the manufacture's instructions. Briefly, media and serial dilutions of PGE_2 standards were incubated at room temperature for 18 h in wells coated with PGE_2 antiserum and acetylcholinesterase-labeled tracer. The reaction mixture was decanted, and the wells were rinsed with wash buffer, then 200 ml of Ellman's reagent, containing substrate for the acetylcholinesterase. The enzyme reaction was carried out for 90 min with slow shaking at room temperature. Then the plates were read at a 415 nm, with the use of the scanning spectrophotometer described above. PGE_2 concentration is expressed in ng/ml/mg of cell protein (bicinchoninic acid protein assay; Pierce).

Statistical Methods. The results are presented as representative experiments or as mean ± SEM. Significance was analyzed with the GRAPHPAD INSTAT program, with the use of ANOVA completed by Dunnett or Dun multiple comparisons posttest, according to the number of experiments. $P < 0.05$ is considered significant.

Results

Four days of exposure to ASA, SA, APAF, or caffeine greatly reduces the number of rapidly proliferating mIMCD-3 cells, as measured by LSC (Fig. 1*A*) or MTT (Fig. 1*B*). ASA, SA, or APAF at a concentration of 0.5 mM reduces the cell number to ≈40–80% of control, and 2.0 mM reduces the cell number to 20% of control or less. Caffeine is less effective, having little effect up to 1.0 mM.

We also attempted to determine whether the drugs might be more toxic in combination than singly. In screening experiments we tested the combinations at several different concentrations of each drug. In general, the combinations are at least partially additive, as demonstrated by the example in Fig. 2*A*, which is not surprising. Of special interest, however, one combination, that of APAF plus caffeine, has a particularly large synergistic effect (Figs. 1*A* and *B* and 2*A*).

SA and APAF have less effect on confluent cells than on rapidly proliferating ones. Although 2.0 mM APAF or SA decreases the number of proliferating cells by 80% or more (Fig. 1), the same concentration of SA does not decrease the number of confluent cells significantly, and 2.0 mM APAF decreases the number of confluent cells by only ≈30% (Fig. 2*B*). Caffeine at a concentration of 2.0 mM does not decrease the number of confluent cells significantly. However, 2.0 mM caffeine plus 2.0 mM APAF decreases cell number by ≈90% (Fig. 2*C*). Thus, the toxic effects of APAF and caffeine are synergistic in confluent as well as in rapidly growing mIMCD3 cells.

A decrease in the number of proliferating cells can occur either because the cell cycle is slowed or because cells die. The following experiments were intended to investigate these possibilities.

Cell death by apoptosis produces apoptotic bodies, each containing a small fraction of the DNA from a nucleus. These appear as a subdiploid peak in flow cytometry. When the rapidly proliferating mIMCD3 cells are exposed to 2 mM SA or 0.5 mM

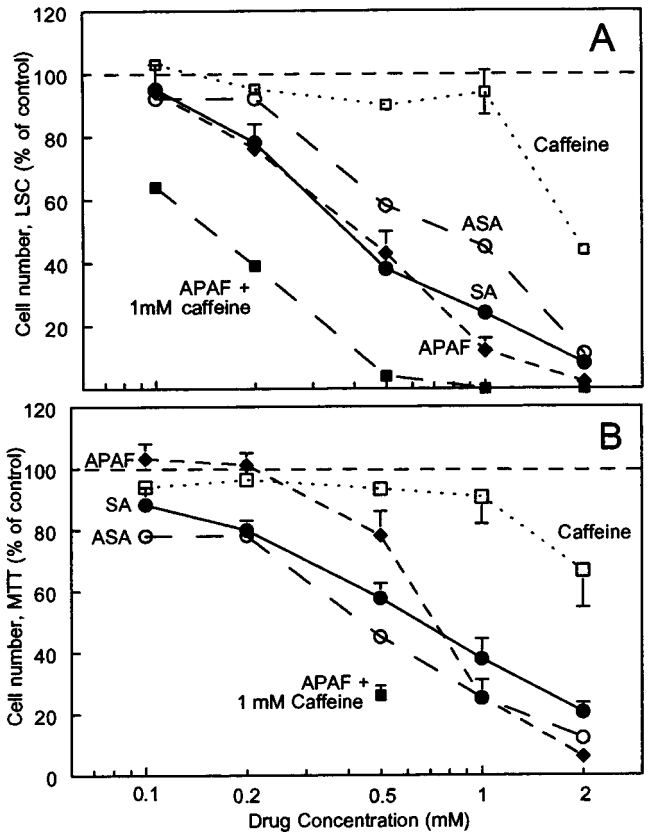

Fig. 1. Effect of APAF, ASA, SA, caffeine, or APAF plus 1 mM caffeine on the number of rapidly proliferating mIMCD3 cells, measured by LSC (*A*) or MTT (*B*). The cells were exposed to the drugs for 3–4 days. Each point represents the mean of two to four (*A*) or two to six (*B*) independent experiments at each concentration.

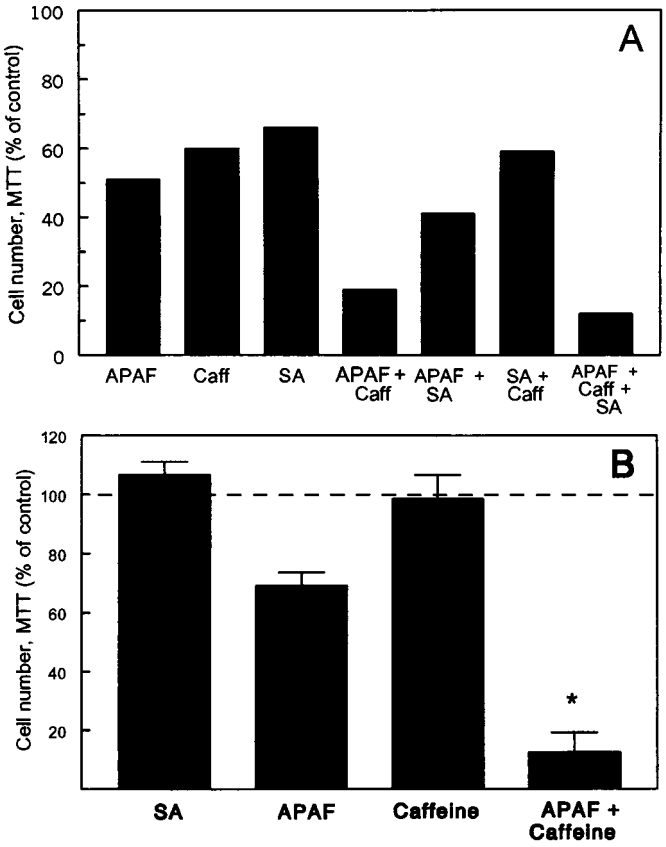

Fig. 2. (*A*) Effects of combinations of 0.1 mM SA, 0.5 mM APAF, and/or 1.0 mM caffeine for 4 days on rapidly proliferating mIMCD3. The cell number was measured by MTT after 4 days of exposure to the drugs. (*B*) Effects of SA, APAF, and caffeine on confluent mIMCD3 cells. The concentration of each drug was 2.0 mM. After 4 days of exposure to the drugs, the cell number was measured by MTT (mean ± SEM, $n = 4$, *, $P < 0.05$ versus APAF alone). FBS was present during the experimental period in two experiments, but not in the other two. Because the results do not differ, they are combined.

APAF the percentage of particles in the subdiploid peak increases greatly, which is consistent with cell death from apoptosis (Fig. 3).

However, the subdiploid peak is more distinct with SA than with APAF (Fig. 3). With 0.5 mM APAF there are a substantial number of particles whose DNA staining is reduced to less than that of cells in G_1 but is still much greater than that of particles in the subdiploid peak (Fig. 3). This pattern of DNA staining is also evident by LSC. With the use of LSC, we located and photographed cells on the slide from that region of a cytogram. The nuclei of those cells are swollen and pale compared with control cells in G_1 (Fig. 3), consistent with APAF killing cells by oncosis as well as apoptosis.

To confirm how APAF kills the cells, we examined them by transmission electron microscopy. When rapidly proliferating mIMCD3 cells are exposed to 2 mM APAF for 36 h, two types of dying cells appear (Fig. 4). Transmission electron microscopy shows both early and late stages of apoptosis (Fig. 4*B*). In the early stage of apoptosis there is limited cell shrinkage and nuclear condensation, whereas in the late stage of apoptosis, not only are the cells contracted, but the nuclear condensation is advanced and apoptotic bodies appear. The oncotic cells are swollen (Fig. 4*C*), especially their nuclei, and their nucleoli are degraded.

We analyzed cell cycle by flow cytometry and LSC to test for NSAID-induced changes that might decrease cell number by slowing proliferation. No change in the cell cycle was apparent after ASA, SA, or caffeine (Fig. 3). However, APAF greatly reduced the percentage of cells in early G_1 and in G_2/M, while increasing the percentage in late G_1 and early S (Fig. 3 and inset in Fig. 4*C*), consistent with alteration of the cell cycle by APAF, but not by the other drugs individually.

NSAIDs generally inhibit COX. To determine the role of COX inhibition in the direct toxicity of NSAIDs for IM cells, we tested the effects of indomethacin, a nonspecific COX inhibitor, and NS398, a specific COX-2 inhibitor. A concentration of 5 μM or more of either drug essentially completely inhibits the COX activity of rapidly proliferating mIMCD3 cells, as shown by reduced PGE_2 production (Fig. 5*A*). These drugs also reduce the number of rapidly proliferating mIMCD3 cells (Fig. 5*B*), but only at concentrations well above those that inhibit COX activity. Thus, 5 μM indomethacin strongly inhibits COX activity (Fig. 5*A*), but 50 μM indomethacin does not affect cell number (Fig. 5). Similarly, 0.1 μM NS398 strongly inhibits COX activity, but 2.0 μM does not affect cell number (Fig. 5). Results of experiments in which cell number was counted by LSC were essentially the same (data not shown). Evidently, these COX inhibitors can be directly toxic to mIMCD3 cells, but inhibition of COX activity, alone, is not sufficient to produce this effect. Caffeine does not potentiate the effect of indomethacin. After 4 days of 5.0 μM indomethacin plus 1.0 mM caffeine, the cell number is 98% of control (LSC, $n = 2$).

Discussion

NSAIDs and Caffeine Are Directly Toxic to IMCD Cells in Culture. Our results demonstrate that the NSAIDs ASA, SA, APAF, indo-

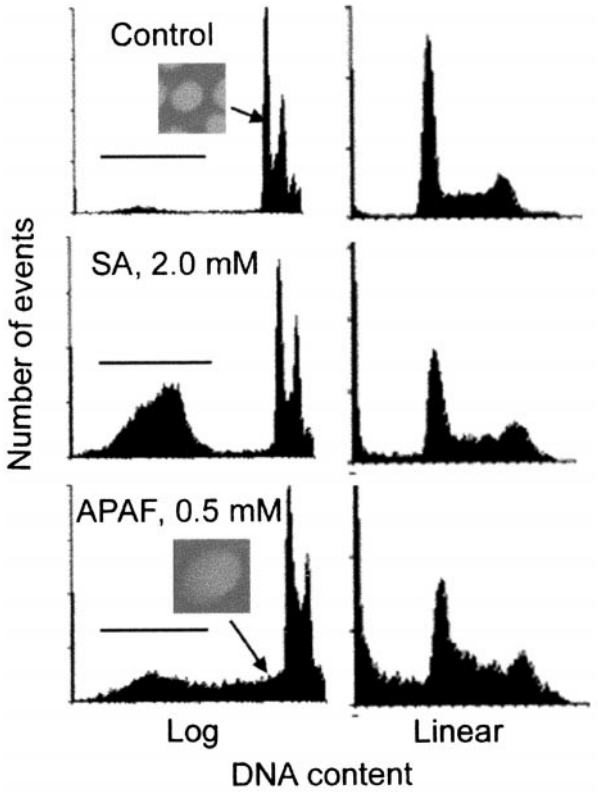

Fig. 3. Flow cytometry of rapidly proliferating mIMCD3 cells treated with SA or APAF for 4 days. Both drugs increase the percentage of particles in a subdiploid peak (under the bars), which are apoptotic bodies. In addition, APAF compared with SA or control results in the subdiploid DNA region having a greater number of cells with DNA content closer to, but still below, the G_1 peak. The nuclei in those cells are intact and are swollen compared with controls (*Insets*, photographed in a separate experiment analyzed by LSC and with a concentration of 2.0 mM APAF).

methacin, and NS-398 are directly toxic to mIMCD3 cells in tissue culture, as is caffeine. Excessive ingestion of NSAIDs and caffeine has been associated with chronic renal failure (1). In what follows we discuss the possibility that these drugs might directly damage renal medullary cells *in vivo*, contributing to analgesic-associated renal disease. We will consider (*i*) the role of COX inhibition in the observed effects, (*ii*) comparison of the drug concentrations that are directly toxic in tissue culture with concentrations that might occur in renal medullas *in vivo*, and (*iii*) possible mechanisms of toxicity. We conclude that direct toxicity to renal medullary cells *in vivo* is a plausible, if unproved, factor contributing to the renal toxicity of NSAIDs taken in excess and that the small population of renal inner medullary cells that are proliferating may be particularly vulnerable, explaining why only very high doses of the drugs, taken over a very long time, lead to chronic renal failure.

Role of COX Inhibition. The mechanism of analgesic-associated kidney disease remains uncertain despite numerous investigations. The analgesics that have been implicated all inhibit COX to varying degrees, although APAF is relatively weak in this regard (19). Many of the theories of the mechanism of analgesic-associated kidney disease involve one or more of the numerous actions of COX in the kidney. Therefore, we were interested in determining whether COX inhibition *per se* might be directly toxic to renal cells in tissue culture. Numerous investigations related to cancer therapy have shown that NSAIDs can be directly toxic to cells. NSAIDs inhibit the proliferation of some

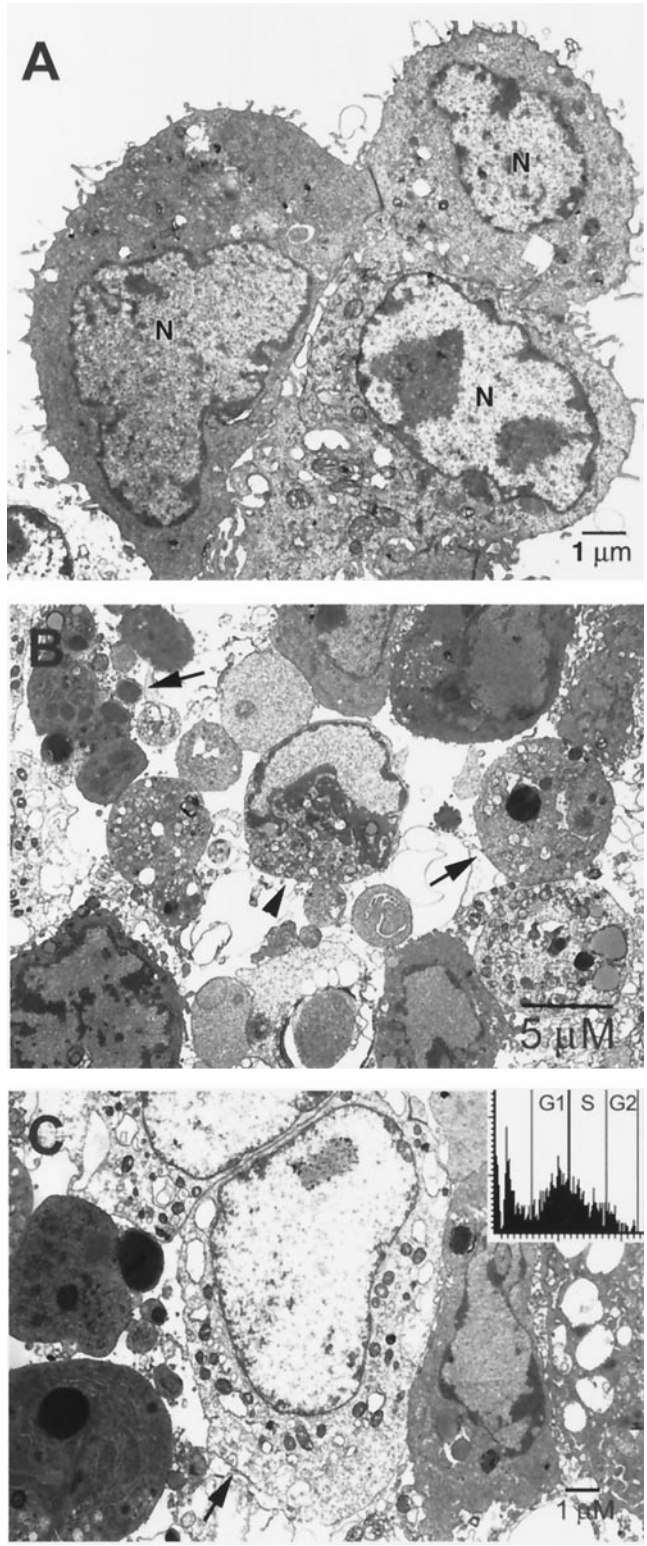

Fig. 4. Electron transmission microscopy of mIMCD3 cells. (*A*) Control. (*B* and *C*) Cells exposed to 2 mM APAF for 36 h. *B* shows both early (arrowhead) and late stages (arrows) of apoptosis. In the early stage of apoptosis there is limited cell shrinkage and nuclear condensation, and in the late stage of apoptosis not only are the cells contracted, but the nuclear condensation is advanced and apoptotic bodies appear. *C* shows both an oncotic cell and several apoptotic cells. The oncotic cell (*Center*) has a swollen nucleus and a degraded nucleolus. The apoptotic cells have greatly condensed nuclei. (*Inset*) A cytogram by LSC with 2 mM APAF. Most cells are arrested in late G_1 and early S. Moreover, there are many oncotic cells whose nuclei have subdiploid DNA staining.

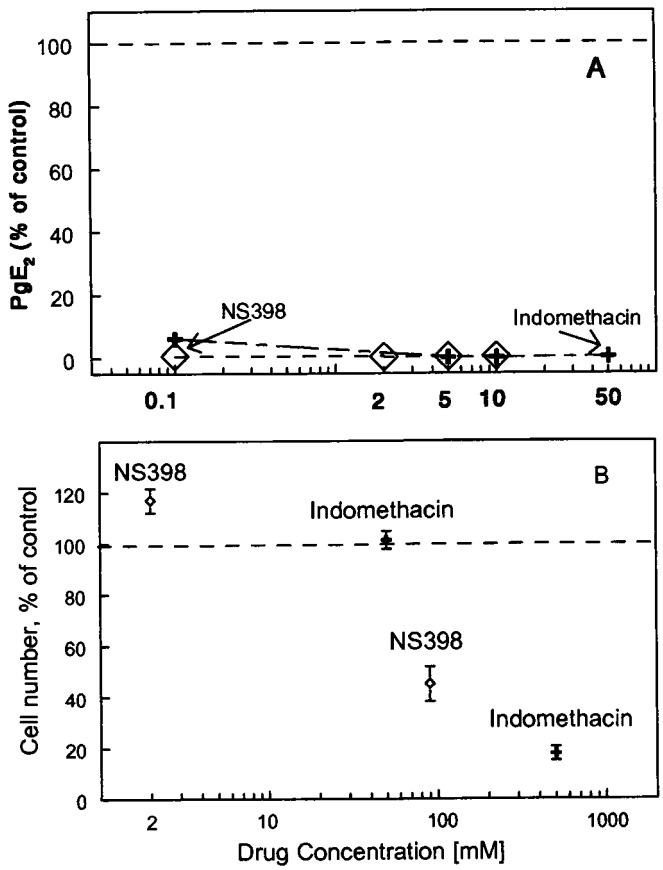

Fig. 5. (*A*) Representative experiment showing the effect of indomethacin (+) or NS398 (◇) on the COX activity of mIMCD-3 cells. COX activity was estimated from the rate of appearance of PGE_2 in the medium. Rapidly proliferating mIMCD-3 cells were exposed to indomethacin or NS398 for 4 days. Arachidonic acid (5 μM) was added during the last 24 h. (*B*) A representative experiment demonstrating the effects of indomethacin or NS398 on the number of rapidly proliferating mIMCD3 cells. The cell number was estimated by MTT assay. Results of at least one experiment for each drug concentration, measuring with MTT, and at least one other experiments measuring with LSC (not shown) were essentially the same. Note that 2 mM NS398 and 50 mM indomethacin do not decrease cell number, despite the complete inhibition of COX.

tumorigenic cells and kill them (11), which has led to numerous studies of the mechanisms involved. Both COX-dependent and independent mechanisms have been identified (10, 11).

In our study of mIMCD3 cells, we find that the COX inhibitors indomethacin and NS-398 substantially reduce the number of rapidly proliferating mIMCD3 cells, but only at concentrations of the drugs that are an order of magnitude or more greater than needed to inhibit COX. We conclude that COX inhibition *per se* is not sufficient to produce toxicity under these conditions. Along this line, we are unaware of evidence that COX inhibition *per se* correlates with analgesic-associated kidney disease. Modern COX inhibitors are tolerated at dosages that give very effective chronic inhibition of COX, but it is not apparent that this chronic inhibition of COX produces chronic renal disease. It will be of interest and concern to observe whether renal disease occurs after such chronic COX inhibition. This might not happen if COX inhibition *per se* is not sufficient to produce chronic renal disease *in vivo*, just as it is not sufficient to produce toxicity in our tissue cultures.

We next consider whether the concentrations of NSAIDs that are directly toxic to renal medullary cells in tissue culture might occur in renal medullas of persons ingesting large quantities of these drugs and, because COX inhibition *per se* is not sufficient to produce direct toxicity under the conditions of our studies, what some additional mechanisms of toxicity might be.

ASA and SA. It is pertinent to estimate the concentration of salicylate that might be present in inner medullas for comparison with the levels that are toxic to proliferating mIMCD3 cells in tissue culture. Most of ingested ASA is rapidly converted to SA. The serum level of salicylate that is effective in rheumatoid arthritis is 150–300 μg/ml ($\approx$1–2 mM.), and clinical salicylate toxicity appears at 200 μg/ml ($\approx$1.5 mM) (20). The level in rabbit inner medulla was found to be several times higher than that in peripheral plasma during antidiuresis (21). Thus, chronic overingestion of ASA or SA might result in millimolar levels of salicylate in the renal medulla. On this basis it is plausible that concentrations of salicylate that are directly toxic could be present in inner medullas when excessive ASA or SA is chronically ingested. The comparison is complicated, however, by the fact that 50% of salicylate is bound to albumin in plasma (22), whereas the percentage bound should be lower in the media we used, which contain only 10% serum. Nevertheless, we conclude that the concentrations of ASA and SA that were directly toxic to rapidly proliferating mIMCD3 cells in culture are plausibly close to those that might occur in renal medullas *in vivo* after ingestion of excessive amounts of the drugs.

APAF. The therapeutic level is 10–20 μg/ml (66–132 μM), and acute toxicity occurs at $\geq$300 μg/ml ($\geq$2.0 mM) (20), which sets an upper limit on plausible plasma concentrations in patients who chronically ingest excessive amounts. In dogs during antidiuresis inner medullary APAF levels were 4 times higher than in peripheral plasma (21). In comparison, as little as 0.5 mM APAF significantly reduced the number of rapidly proliferating mIMCD3 cells (Fig. 1). Thus, the concentrations of APAF that are directly toxic to rapidly proliferating mIMCD3 cells in culture might occur in renal medullas *in vivo* when excessive amounts of the drug are ingested chronically.

Acute overdoses of APAF cause acute and sometimes fatal liver damage (23). The toxicity is caused by a minor metabolic product, *N*-acetyl-*p*-benzoquinone imine, that attaches to the hepatic cell membranes and injures the lipid bilayer if not neutralized by an antioxidant. Hepatic glutathione appears to be the primary antioxidant that conjugates and neutralizes *N*-acetyl-*p*-benzoquinone imine. The resulting oxidative stress in the cell may ultimately lead to its demise. *N*-acetyl-*p*-benzoquinone imine also binds to cell macromolecules, which can cause cell death (24).

However, APAF has an additional toxic effect that may be more pertinent to the toxicity that we observed. The drug directly inhibits ribonucleotide reductase, which reduces cell growth by stopping DNA replication (25). Then, the relative number of cells in the S phase increases (25), as we observed after exposure of mIMCD3 cells to APAF (Figs. 3 and 4). In the process DNA is damaged, leading to sister chromatid exchange and chromosomal aberrations. APAF also inhibits nucleotide excision repair (26). These genotoxic effects of APAF could contribute to the development of the uroepithelial tumors that accompany analgesic-associated kidney disease.

Caffeine. The correlation of the caffeine content of analgesic mixtures with subsequent renal failure can be accounted for by the mental stimulating effect of caffeine, which could contribute substantially to the initiation and perpetuation of high cumulative intake of the mixtures (5). However, caffeine is also known to play a more direct role in exacerbating the toxic effects of other agents, at least in cell culture. Therefore, we are led to consider whether chronic ingestion of excessive amounts of

caffeine could raise its level in the renal inner medulla enough to produce such direct effects. Many cases of fatal acute caffeine poisoning are associated with plasma concentrations of 0.5–2.0 mM (23), which is the level that affected the mIMCD3 cell number in our experiments. It is unclear, however, how high plasma caffeine is during chronic excessive intake and whether caffeine concentration could be higher in the inner medulla than in peripheral plasma during antidiuresis, like APAF and salicylate concentrations are. Thus, it is conceivable that the effects that we observed in tissue culture and other effects discussed below occur in renal medullas *in vivo*.

Caffeine has long been known to have numerous actions (27), including (*i*) inhibition of phosphodiesterases, thereby increasing intracellular cAMP; (*ii*) direct effects on intracellular calcium concentration; (*iii*) indirect effects on intracellular calcium concentrations via membrane hyperpolarization; and (*iv*) antagonism of adenosine receptors. In addition, it has recently become apparent that caffeine also influences multiple pathways involved in the cellular response to DNA damage. It reduces DNA damage-induced cell cycle arrest in G_1, S, and G_2/M, abolishing the G_2/M checkpoint by inhibiting ATM kinase activity (28). Caffeine also blocks p53 activation in response to DNA damage and blocks the repair of DNA damage (12). The result is that caffeine potentiates the lethal effects of ionizing radiation, which could be useful for cancer therapy. Unfortunately the concentration of caffeine that is required is too high for clinical use.

Drug Interactions. Analgesic-associated renal disease has most often been observed after the excessive ingestion of combinations of drugs (1). Therefore, we tested the effects of combinations of SA, APAF, and caffeine. Although a high concentration of caffeine (2 mM) reduces the number of proliferating mIMCD3 cells significantly, the effect is relatively small (Fig. 1). Interestingly, however, caffeine strongly potentiates the toxicity of APAF (Figs. 1 and 2). The mechanism could be related to the effects of on both DNA damage and repair, but we are unaware of any direct experimental evidence for this connection. Nevertheless, based on these observations, we speculate that interactions between caffeine and APAF could contribute to analgesic-associated kidney disease and help to explain why this form of chronic renal failure has frequently been associated with ingestion of mixtures of these drugs.

Role of Proliferation. When the mIMCD3 cells are confluent and their rate of proliferation decreases, SA and APAF are less toxic (Fig. 2) than when the cells are rapidly proliferating (Fig. 1). Therefore, inner medullary cells in tissue culture are much more susceptible to toxicity of NSAIDs when they are proliferating than when they are quiescent. The rate of proliferation of rat renal inner medullary cells *in vivo* is very slow, as indicated by the finding of very few cells that express proliferating cell nuclear antigen in rat renal inner medullas (unpublished observation). Extrapolating from these observations, we propose that the few proliferating cells that are slowly replacing existing ones might be killed by excessive ingestion of NSAIDS. This conclusion could explain why only high doses taken for years are toxic. Only the few proliferating cells that are slowly replacing existing ones *in vivo* might be killed, reducing cell number by attrition.

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