Journal of Neuro-Oncology **46:** 215–229, 2000.

Laboratory Investigation

Acetaminophen selectively reduces glioma cell growth and increases radiosensitivity in culture

Diana Casper[1], Rukmani Lekhraj[1], UmaRani S. Yaparpalvi[1], Ann Pidel[1], Wainwright A. Jaggernauth[1], Peter Werner[2,4], Silke Tribius[3], John Del Rowe[3] and Patrick A. LaSala[1]
[1]*Department of Neurological Surgery,* [2]*Department of Neurology,* [3]*Department of Radiation Oncology, Montefiore Medical Center, Bronx;* [4]*Department of Neurology, Beth Israel Medical Centre, New York, NY, USA*

Key words: NSAIDs, acetylsalicylate, acetaminophen, ibuprofen, toxicity, radiation, glioma

Summary

Glioblastoma multiforme (GBM) is a highly lethal brain cancer. Using cultures of rodent and human malignant glioma cell lines, we demonstrated that millimolar concentrations of acetylsalicylate, acetaminophen, and ibuprofen all significantly reduce cell numbers after several days of culture. However, their mechanisms of action may vary, as demonstrated by (1) differences in the morphological changes produced by these compounds; (2) varied responses to these drugs with respect to toxicity kinetics; and (3) respective rates of cell proliferation, DNA synthesis, and mitotic index. We studied the effects of acetaminophen on relative cell number further. Evidence is presented that acetaminophen induced cell death by an apoptotic mechanism after a brief burst of mitosis in which cell numbers increased transiently, followed by a reduction in cell number and an increase in DNA fragmentation, as evidenced by terminal deoxytransferase-mediated dUTP-biotin nick end labeling (TUNEL) analysis. Using cultures of adult human brain and embryonic rat brain, we demonstrated that glioma cells were several-fold more sensitive to acetaminophen than normal brain cells in culture. Finally, subtoxic doses of acetaminophen increased the sensitivity of the human glioma cells in culture to ionizing radiation. Taken together, these results suggest that acetaminophen may prove to be a useful therapeutic agent in the treatment of human brain tumors.

Introduction

Acetylsalicylate, acetaminophen, and ibuprofen are three over-the-counter medications which are widely and regularly used by much of the world for their analgesic, antipyretic, and anti-inflammatory properties. They are reported to be the most widely used drugs in medicine, and it is believed that their therapeutic effects are due to the inhibition of prostaglandin biosynthesis. Interest in the relationship between anti-inflammatory agents and cancer arose in the late 1970s when researchers found that concentrations of several prostaglandins were elevated in human tumors of the colon, lung, and breast, and that prostaglandin concentration positively correlated with tumor size (see [1] for review and references). For these reasons, all of these drugs have also been investigated with respect to cancer risk in humans [2]. In the early 1990s, several retrospective, prospective, and case-control studies which indicated that regular long-term acetylsalicylate users had a lower incidence of colorectal cancer were published [3]. Other acetylsalicylate-like, non-steroidal anti-inflammatory drugs (NSAIDs) had similar effects, but acetaminophen use did not show any correlation with risk for colon cancer [4,5]. A recent report demonstrated a significant negative correlation between ovarian cancer and acetaminophen use [6], and a small clinical trial of high dosage acetaminophen treatment reported some regression of other types of advanced cancer [7]. However, studies have shown that acetaminophen use positively correlates with risk for renal pelvic cancers [8,9], although this finding was not reproduced in later studies [10]. Taken together, these studies suggest that acetylsalicylate,

ibuprofen, and acetaminophen may have therapeutic potential in the treatment of certain kinds of cancer, but the interpretation of epidemiological studies may be complicated by selection biases and other factors [11].

Experimental rodent studies support and extend some of the observations made in humans. For example, it has been demonstrated that acetylsalicylate-like NSAIDs could inhibit chemically induced adenomas and early carcinomas of the rat colon. They reduced the incidence (% of animals), multiplicity (number per animal), and size (volume or weight) of tumors *in vivo* [12–14]. In some studies, these compounds were effective even if they were introduced after initial tumor growth. Inhibition of tumor growth and/or reduction of tumor size have also been demonstrated for fibrosarcomas and mammary tumor cells in animal models [15,16]. Ibuprofen was shown to significantly reduce the size of C6 glioma spheroids implanted into rat brain [17]. However, these studies did not definitively show whether these compounds had direct or secondary effects due to immune system stimulation or inhibition of angiogenesis, a process in which prostaglandin synthesis has been shown to be an integral component [18].

Cell culture is particularly advantageous in dissecting out the differences between direct and indirect mechanisms of action. These systems isolate a particular cell type from the body and they can be grown in a large number of replicates (sister cultures). Compounds under investigation can then be added directly, with no routing through the circulatory system or stimulation of the immune system. Studies carried out *in vitro* have indicated that NSAIDs such as acetylsalicylate, indomethacin, sulindac, and acetaminophen may also have a direct mechanism of action on tumor cells [19–21], but their mechanisms of action and their specificity for certain cell types are not well understood. Furthermore, no experiments have been performed to date addressing the direct effects of acetaminophen on cancer cells *in vitro*.

In this report we compare the effects of acetylsalicylate, acetaminophen, and ibuprofen on human and rodent glial tumor cell numbers *in vitro*. The mechanisms of these effects are further characterized with respect to dose, kinetics, cell cycle effects, and morphology. Finally, the mechanism of toxicity is further characterized for acetaminophen with respect to apoptotic cell death, selective vulnerability, and radiosensitivity.

Materials and methods

Cell culture

SNB-19 cells, a human glioblastoma cell line [22], and the C6 rat glioma cell line, derived from a N-nitrosomethylurea-induced tumor [23], were used for these studies. Cells were maintained and passaged in RPMI 1640 culture medium containing a non-hydrolyzeable glutamine analog (Life Technologies, Grand Island, NY), supplemented with 15% heat inactivated fetal bovine serum (Life Technologies). Experiments were performed after reducing the serum concentration to 5% for several hours or overnight. Human non-tumor cells were obtained from temporal lobe tissue excised in surgical procedures for epilepsy at the Montefiore Medical Center. A small piece of tissue was trypsinized, mechanically dissociated, and grown in culture for several passages. Rat primary glial cultures were established from E16 embryonic rat forebrain by dissection, mechanical dissociation, plating, and passing several times in order to eliminate neuronal elements.

Treatment with drugs

Acetylsalicylate (aspirin), acetaminophen, and ibuprofen were obtained from Sigma (St. Louis, MO). Compounds were dissolved in ethanol to make a stock solution at least 100 times the concentration desired, and diluted in culture medium for subsequent incubations. Ethanol itself (maximum concentration 1%) had no effect on any experiment described here, and did not potentiate the effects of acetaminophen (see Discussion). Replicate cultures were incubated in the presence or absence of drugs at designated concentrations for various times (see figure legends), and analyzed thereafter.

BrdU labeling and MC6 immunocytochemistry

Treated cell cultures were incubated with 1 μM BrdU (bromodeoxyuridine; Amersham Life Science Inc., Arlington Heights, IL) for 3.5 h, and fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 10 min. After washing with phosphate-buffered saline (PBS; 140 mM NaCl 10 mM sodium phosphate, pH 7.4) and permeabilizing with 90% ethanol/1% glacial acetic acid (−20°C) for 6 min,

cultures were washed and incubated for 30 min with anti-BrdU antibody (Amersham), diluted 1 to 5. Peroxidase immunocytochemistry was performed with the mouse Vectastain and diaminobenzidine (DAB) visualization kits (Vector Laboratories, Burlingame, CA), as per manufacturers protocols. The MC6 antibody was a gift of Dr. Peter Davies, Department of Pathology, The Albert Einstein College of Medicine. MC6 immunocytochemistry was performed in the same manner, starting with the fixation step. MC6 cell culture supernatant was diluted 1 to 10.

Cell counting and photomicroscopy

Nikon TMS inverted and Optiphot microscopes were used at 10× and 20× magnifications for counting and photomicroscopy. Cell numbers were quantified using phase contrast magnification (for total cell counts), and bright field illumination (for immunolabeled cell counts). Cell counts were made of two consecutive microscopic fields under 10× magnification in quadruplicate cultures in each treatment group. Data was analyzed by Student's *t*-test or analysis of variance (ANOVA), followed by Fisher's PLSD *post-hoc* test using the Statview computer program (Abacus Concepts, Inc., Berkeley, CA). The validity of this method was confirmed by experiments in which cells were treated in an identical fashion, but instead of counting representative fields of adherent cells, cultures were trypsinized and cells were counted with a hemacytometer. Similar results were obtained using both methods.

TUNEL analysis

Cells were plated on chamber slides, treated with 1 mM acetaminophen for various times, and fixed with 4% paraformaldehyde. Digoxigenylated dUTP end labeling was performed as previously described [24] and modified as follows: after permeabilization with 90% ethanol/1% glacial acetic acid at −20°C for 5 min, DNAse I was added to positive control wells (0.2 μg/ml DNAse I in 10 mM NaCl, 10 mM Tris–HCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 25 mM KCl, pH 7.4), for 15 min at 37°C. All wells were then equilibrated in a buffer containing 30 mM Tris and 140 mM cacodylate for 5 min, and then incubated with 0.075 nmol digoxigenin-conjugated dUTP, 0.05 U/μl terminal deoxynucleotide transferase (TdT; Boehringer Mannheim, GmbH), and 2.5 mM $CoCl_2$ in the same buffer at 37°C for 30 min. Negative control wells were incubated in the same solution without TdT. The reaction was stopped with Oncor's stop/wash buffer (Cat #8711-4), and immunocytochemistry was subsequently performed with 5 μg/ml sheep anti-digoxigenin Fab fragments (Boehringer Mannheim), biotinylated anti-sheep secondary antibody (diluted 1 to 200, Vector Labs), alkaline phosphatase–streptavidin (diluted 1 to 200, Amersham), and Fast Red (Sigma).

Radiation sensitivity

Radiosensitivity was assessed by clonogenic survival assay, in which cell survival was scored on the basis of colony formation subsequent to ionizing radiation under culture conditions [25]. Monolayer cultures were trypsinized in exponential growth phase and counted. Cells were plated at various densities for different doses of radiation, with the least number of cells being plated for the control set (no radiation). After 3 h, the medium was changed to medium which contained either 0.5 mM acetaminophen or vehicle. After 4 h, irradiation was performed at room temperature. Radiation was administered with a 6 MV linear accelerator (LINAC 6, Varian Oncology Systems, Palo Alto, CA) at a dose rate of 2.5 Gy/min. After 2 weeks of incubation, cultures were stained with 0.5% crystal violet (DIFCO Laboratories, Detroit, MI), and colonies containing more than 50 cells were counted. Survival fractions were calculated to be the number of clones observed for each radiation dose divided by cells plated multiplied by the plating efficiency (the number of clones divided by the total cells plated in the absence of radiation).

Results

Acetylsalicylate, acetaminophen, and ibuprofen decrease the relative numbers of human and rodent glioma cells in a dose- and time-dependent manner

Dose response experiments established the relative potencies of acetylsalicylate, acetaminophen, and ibuprofen to produce decreases in cell number for C6 and SNB-19 glioma cells relative to untreated

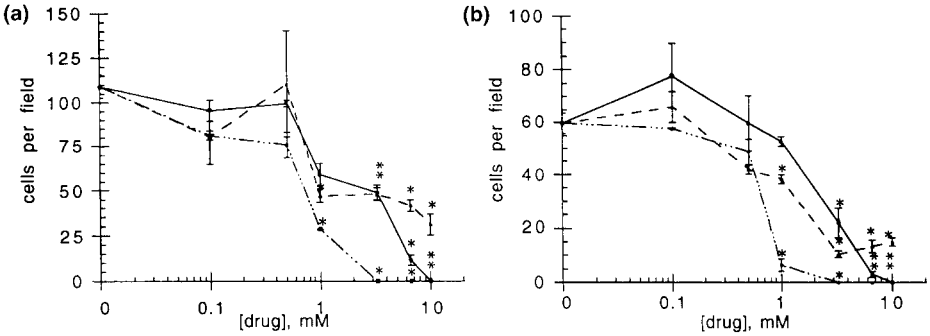

Figure 1. Effects of acetylsalicylate, acetaminophen, and ibuprofen on rat and human glioma cells in culture: dose response. Cultures of SNB-19 (a) and C6 (b) glioma cells were treated with varying concentrations of acetylsalicylate (solid lines), acetaminophen (dashed lines), or ibuprofen (dashed and dotted lines) shown above for two days. Average relative cell numbers (± s.e.m.) were obtained by counting cells in two microscopic fields for quadruplicate cultures under 100× magnification. Asterisks (*) represent significant differences between acetylsalicylate, acetaminophen, or ibuprofen treatments and untreated control cultures ($p < 0.03$), as determined by ANOVA followed by Fisher's PLSD *post-hoc* analysis.

cultures. In Figure 1, data from one such experiment is presented. Replicate cultures were incubated in the presence or absence of various millimolar concentrations of drugs in culture medium. Results demonstrate a dose-dependent decrease in relative cell numbers for both human (panel a) and rat (panel b) gliomas in response to increasing drug concentrations. At concentrations of less than 1 mM, no significant decreases in cell numbers were apparent after two days of treatment with any drug. However, at 1 mM concentrations, significant reductions in cell numbers were seen after treatment with both acetaminophen and ibuprofen, with ibuprofen being more potent for both SNB-19 and C6 cell lines. At 3.3 mM concentrations, no viable cells remained after treatment with ibuprofen, whereas acetylsalicylate and acetaminophen reduced cell numbers by 55% from that of control cultures (from 108 to 48 for both drugs). Higher concentrations of all drugs had more marked effects. We observed that concentrations of 1 mM and higher of acetylsalicylate and ibuprofen acidified the culture medium, as indicated by the phenol red pH indicator. However, at a concentration of 1 mM, acetylsalicylate had the least potent effects, suggesting that medium acidification was not the prevailing mechanism accounting for the decrease in cell numbers. In panels (a) and (b) it can be also seen that although acetaminophen produced a dose-dependent effect on cell numbers, even the maximal concentration (10 mM) of acetaminophen never produced complete elimination of cells after two days.

An experiment comparing the effects of equimolar doses (1 mM) of acetylsalicylate, acetaminophen, and ibuprofen on SNB-19 and C6 glioma cells with respect to time is presented in Figure 2 in panels (a) and (b), respectively. Each line represents cell survival with time in response to treatments by various drugs. After one day, significant differences in cell numbers were observed between untreated cultures and those treated with acetaminophen or ibuprofen, but not acetylsalicylate, for both cell lines. However, no absolute reduction in cell number was seen at this time compared with the number of cells present on the day of plating. After two days, however, ibuprofen treatment reduced the number of cells in both human and rodent cell lines to below the number plated (53% and 22% of original cells plated, respectively). Acetylsalicylate and acetaminophen, in contrast, never actually decreased the number of cells over the time period they were measured, but significantly decreased the relative number of cells compared with untreated cultures. In the experiment represented in this graph, acetylsalicylate and acetaminophen demonstrated equally potent effects on SNB-19 cells, but were differentially effective for C6 cultures, with acetaminophen producing a greater effect at all time points measured. To ensure that cell counts after drug treatments represented viable cells, replicate cultures treated with 1 mM concentrations of acetylsalicylate, acetaminophen, or ibuprofen were harvested by trypsinization after 2 days and viable cells were counted. Reductions of 14%, 40%, and 63% in cell

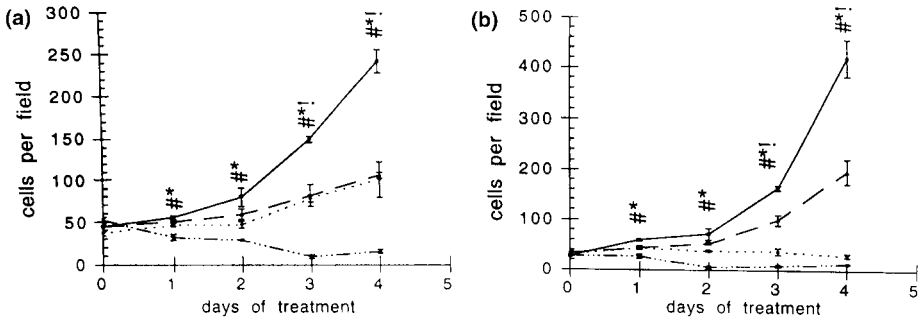

Figure 2. Effects of acetylsalicylate, acetaminophen, and ibuprofen on rat and human glioma cells in culture: time course. Cultures of SNB-19 (a) and C6 (b) glioma cells were treated with 1 mM concentrations of acetylsalicylate (dashed lines), acetaminophen (dotted lines), or ibuprofen (dashed and dotted lines). Growth curves of untreated cultures are represented by solid lines. Average relative cell numbers were obtained (± s.e.m.) by counting cells in two microscopic fields for quadruplicate cultures under 100× magnification. Symbols represent significant differences between cell numbers in aspirin (!), acetaminophen (*), and ibuprofen (#)-treated cultures and untreated controls ($p < 0.0005$) as determined by ANOVA followed by Fisher's PLSD *post-hoc* analysis.

viability were obtained after treatment with the three drugs, respectively.

Acetaminophen stimulates mitosis, but inhibits DNA synthesis

From the experiments described above, although it was apparent that ibuprofen was toxic, killing cells in a dose- and time-dependent manner, it was not possible to distinguish whether acetylsalicylate and acetaminophen decreased the rate of proliferation or induced cell death. In order to further investigate whether the drugs were toxic or anti-mitotic, cells were labeled with two markers for different phases of the cell cycle: BrdU to provide an index of DNA synthesis; and MC6, a monoclonal antibody which selectively stains mitotic cells in culture [26]. Cells were incubated for 3.5 h with or without BrdU in the presence of 1 mM concentrations of acetylsalicylate, acetaminophen, and ibuprofen, after which time they were fixed and processed for BrdU and MC6 immunocytochemistry. Total cell counts were also obtained. Results are presented in Figure 3. Micrographs illustrating the objects counted appear to the right of each graph. Interestingly, after this brief incubation period, total cell numbers in acetaminophen- and ibuprofen-treated cultures, but not acetylsalicylate-treated or control cultures, increased two-fold (Figure 3A), in contrast to the relative decreases found with acetaminophen and ibuprofen treatment at longer time points (see Figure 2). Subsequent experiments were performed at this time point to determine whether these drugs were affecting the rate of mitosis before cells died. The number of cells in M-phase are represented in Figure 3B, along with a micrograph illustrating several cells expressing the MC6 epitope (arrows). Acetylsalicylate-treated cultures had the same number of total cells and the same index of mitotic cells per field (MC6+ cells) as control cultures. Acetaminophen treatment produced an atmost two-fold increase in the number of mitotic cells. However, even though total cell numbers doubled in response to ibuprofen treatment, (as they did with acetaminophen treatment), the number of cells expressing the MC6 mitotic epitope fell by 80%. In acetaminophen-treated cultures, the proportion of the cells in M-phase was similar to control and acetylsalicylate-treated cultures, ($\sim$ 3%), reflecting comparable increases in mitosis and cell number. In contrast, the percentage of mitotic cells in ibuprofen-treated cultures fell ten-fold. Taken together, these results suggest that either the kinetics of similar mechanisms are faster with ibuprofen treatment and that the mitotic component is complete by 3.5 h after treatment, or that the mode of action for each drug is different in this system.

In order to further examine cell cycle kinetics, BrdU labeling was performed in cultures treated under the conditions described above. These cells are depicted in a representative micrograph in Figure 3C, in which the black arrow points to a labeled cell, and a hematoxylin-counterstained unlabeled cell is denoted with a white arrow. In contrast to the results described above for M-phase cells, the number of cells in S-phase decreased significantly in response to both acetaminophen

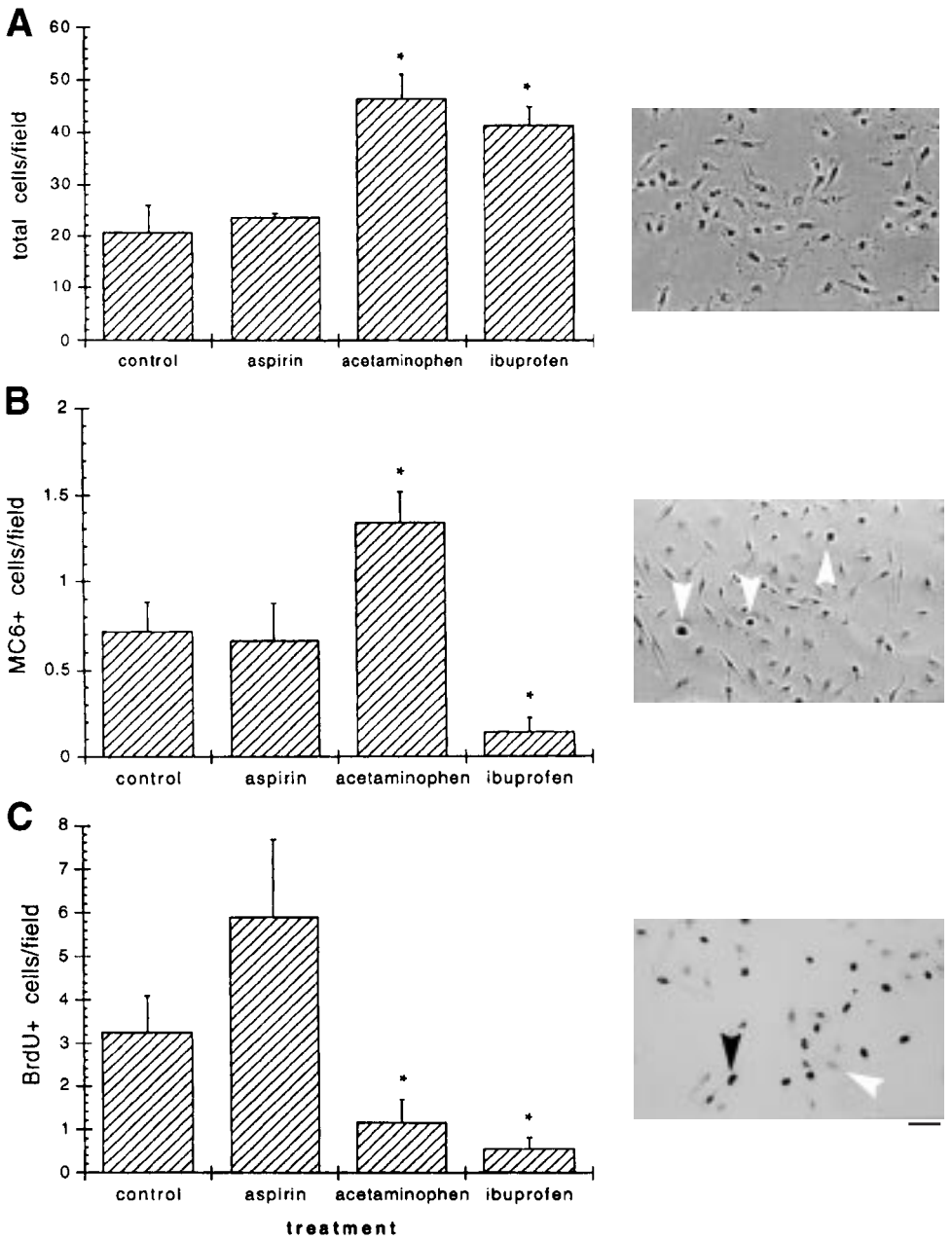

Figure 3. Effects of aspirin, acetaminophen, and ibuprofen on total cell number, mitotic cell number, and the number of cells in S-phase in the SNB-19 glioma cell line. Replicate chamber slide cultures were treated with 1 mM concentrations of each compound. After 3.5 h they were fixed and processed for quantification of (A) total cell numbers, (B) mitotic cell number, using MC6 immunocytochemistry, and (C) cells undergoing DNA synthesis, by incubation with BrdU, followed by fixation and BrdU immunocytochemistry. Bars represent means ($\pm$ s.e.m.) of phase-dark (total) and immunopositive cells (MC6) or nuclei (BrdU), quantified by counting two microscopic fields for quadruplicate cultures under 100$\times$ magnification. Asterisks represent significant differences between treated and control cultures, as determined by ANOVA followed by Fisher's PLSD *post-hoc* analysis ($p < 0.05$). Insets illustrate the materials used for each analysis. Inset for (A): phase-dark SNB-19 cells, (B): MC6-immunoreactive cells (in brown with white arrowheads), usually appearing large and round, and (C): BrdU-labeled nuclei (in brown with black arrowhead). Non-dividing cells are counterstained with hematoxylin (white arrowhead). Scale bar below inset in (C) = 55 μm for all insets.

and ibuprofen treatment. Acetylsalicylate caused an increase, although it was not statistically significant. Therefore, even though acetaminophen and ibuprofen both stimulated cells to divide, they did not stimulate DNA-synthesis. This result confirms the results of previous experiments which demonstrated that cell numbers in acetaminophen-treated cultures cease to increase at later time points because another round of DNA replication failed to take place.

Acetaminophen and ibuprofen produce distinct morphological changes in glioma cells

Acetaminophen and ibuprofen had marked effects on cell morphology, illustrated for SNB-19 cells in Figure 4. In panel (a), control cells are heterogeneous in shape, but the majority of cells appear flat and symmetrical, with vellate cytoplasm and slightly ruffled edges (arrow). Nuclei appear phase-dark, with a phase-bright margin between nuclear and cytoplasmic compartments. In panel (b), cells treated with acetylsalicylate have similar shapes to untreated cells, but the cytoplasm is somewhat more ruffled, with phase-dark edges (arrow). Phase-bright, process-bearing cells are also seen (arrowhead). Acetaminophen treatment produced more marked morphological effects, as illustrated in panel (c). Many cells were found to have a spindle shape (arrow), with several pointed processes extending from various points on the cell body. Other cells had a single fine process extending from the cell body. In contrast, ibuprofen treatment (panel d) resulted in opposite changes in cell shape. The cells became smaller (arrow); there were more phase-bright figures (arrowhead), probably due to nuclear condensation; and many tiny, short, thin processes (microspikes) extended from the cell body. These cells are reminiscent of apoptotic cells, with condensation of cell body, consistent with our observation that this treatment produced the largest relative decrease in cell number.

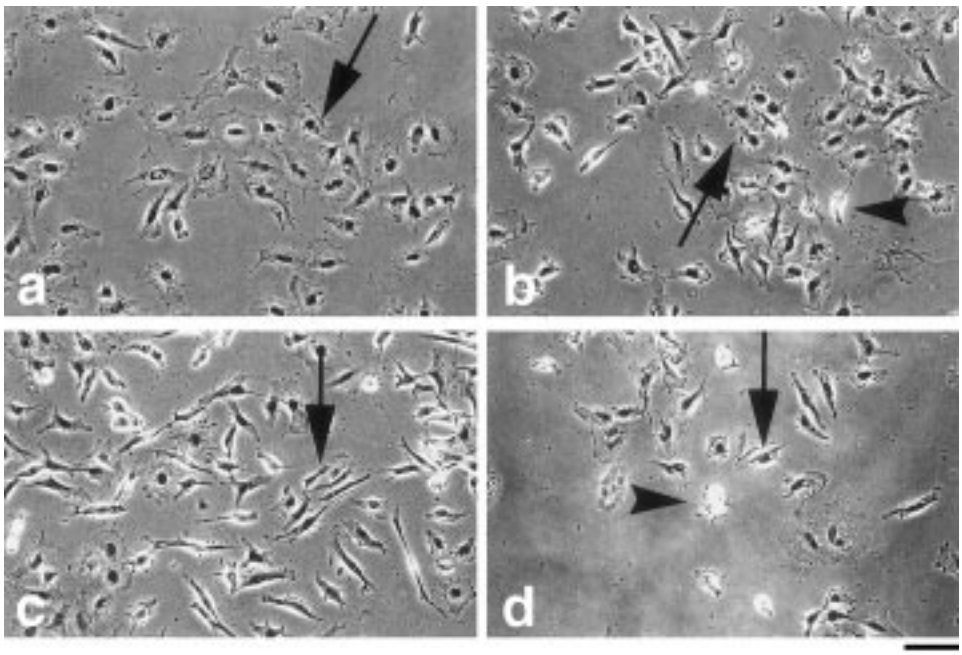

Figure 4. Effects of acetylsalicylate, acetaminophen, and ibuprofen on human SNB-19 glioma cells in culture: morphology. Cultures were treated for two days with either (a) vehicle (1% ethanol) or 1 mM concentrations of (b) acetylsalicylate, (c) acetaminophen, or (d) ibuprofen. Photomicrographs represent typical fields seen under 200× magnification for each treatment. Panel (a): cells displaying the typical morphology seen in culture are indicated by the large arrow, pointing to a group of cells with nuclei in the center of symmetrically arranged vellate cytoplasm. Panel (b): acetylsalicylate-treated cells are similar in size and shape to control cells, but with increased membrane ruffling (arrow). Phase-bright, condensed cellular material is present (arrowhead). Panel (c): arrow points to a cluster of spindle-shaped cells resulting from acetaminophen treatment. Panel (d): ibuprofen treatment produces a decrease in cell number with a reduction in cell size (arrow) accompanied by the appearance of phase-bright cellular debris from which cytoplasmic microspikes extend (arrowhead). Scale bar below panel (d) = 62 μm.

Further characterization of acetaminophen toxicity: acetaminophen induces apoptosis

Experiments demonstrating short term increases, followed by relative decreases in glioma cell number with acetaminophen treatment suggested that acetaminophen was toxic. The mechanism of cell death was further investigated. Since acetaminophen-induced toxicity has been demonstrated to cause DNA-fragmentation and cell death in hepatocytes [27], we examined SNB-19 glioma cultures treated with acetaminophen for evidence of apoptosis. Cultures were incubated with 1 mM acetaminophen and processed for TUNEL after 1, 2, 3 and 4 days of incubation. Results are illustrated in Figure 5, and the appearance of the material analyzed is depicted in the inset, in which a TUNEL-positive cell is shown (arrow) in a field of viable, unlabeled cells. Significant differences in the numbers of apoptotic cells were found between control and acetaminophen-treated cultures at the same time points at which numbers of acetaminophen-treated cells were seen to decrease relative to those of control cultures (Figure 2). In experiments, the apoptotic index (100 times the number of apoptotic cells per field) for control cultures remained within the range of 1–2.5 over the 4 days at which values were measured. In contrast, acetaminophen-treated cultures exhibited up to 8-fold higher rates of apoptosis, with values reaching 16 ± 2.2, significantly higher than that of untreated cultures at 3 out of 4 time points (Student's t-test; $p < 0.05$).

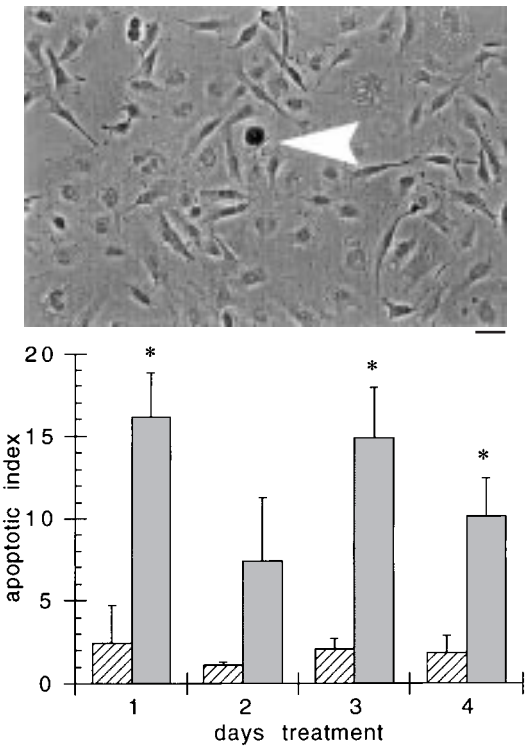

Figure 5. Acetaminophen decreases SNB-19 cell viability by induction of apoptotic cell death. Replicate cultures of SNB-19 cells were treated with 1 mM acetaminophen or control medium for 1, 2, 3 or 4 days followed by fixation and processing for TUNEL analysis. Striped bars: control cultures, stippled bars: acetaminophen-treated cultures (+ s.e.m.). Asterisks denote significant differences between treated and untreated quadruplicate cultures as determined by ANOVA followed by Fisher's PLSD *post-hoc* analysis ($p < 0.05$). Inset: arrowhead points to TUNEL-positive cell, indicative of apoptosis. Scale bar = 44 μM.

Acetaminophen has differential potency on tumor cells compared with non-tumor cells

In order to determine whether or not the effects of acetaminophen on cultured cells were selective for tumor cells, we compared acetaminophen treatment on the SNB-19 cell line with primary human brain glia. Cultures were established from human temporal lobe tissue in the presence of serum, resulting in mixed cultures composed predominantly of astrocytes and mesenchymal cells, as assessed by immuocytochemistry for glial and neuronal markers (data not shown). Cultures were simultaneously treated with various concentrations of acetaminophen and cell numbers were assessed in several microscopic fields under 100× magnification. Results are depicted in Figure 6. Solid and dotted lines represent survival curves with respect to acetaminophen dose for primary cultures (solid line) and SNB-19 glioma cultures (dotted line), respectively (Figure 6a). Although cell numbers decreased in response to acetaminophen treatment in a dose-dependent fashion for both cell types, we observed that for any concentration of acetaminophen added, less tumor cells survived after two days of treatment. For example, in this experiment, a 1 mM concentration of acetaminophen for two days decreased SNB-19 cell number to 38% of untreated cultures, but did not result in any significant change in cell number of primary cultures. In order to produce comparable decreases in non-transformed cell cultures, at least a 3 mM concentration of acetaminophen was required. The same observation was made when comparing rat C6 glioma cells with primary cultures established from embryonic rat forebrain (Figure 6b). The same dose of acetaminophen (1 mM) that produced a 50%

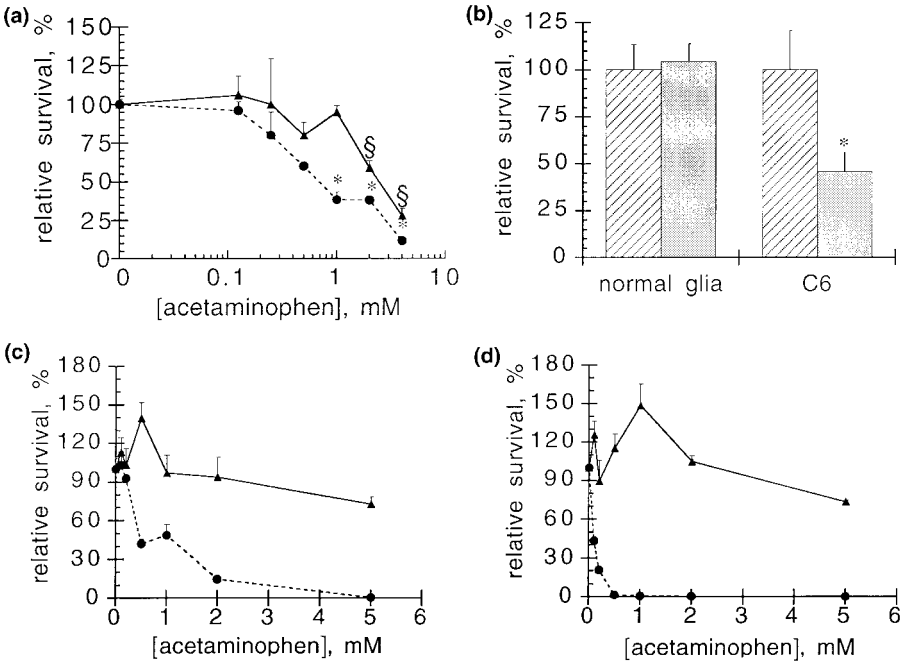

Figure 6. Human and rat glioma cells are more vulnerable to acetaminophen toxicity than primary brain cells in culture. Panel (a): Replicate cultures of both SNB-19 cells and human brain cultures were treated with various concentrations of acetaminophen for 2 days. Relative cell numbers were obtained by counting cells in 2 microscopic fields for quadruplicate cultures at each concentration under 100× magnification. Solid line: primary brain cultures. Dashed line: SNW-19 cultures. Panel (b): Replicate cultures of C6 rat glioma cells and primary embryonic brain cultures were established and treated with 1 mM acetaminophen for 2 days. Cell counts for quadruplicate cultures were obtained, as above. Striped bars: untreated cultures. Stippled bars: acetaminophen- treated cultures. Values represent means (+s.e.m.). Asterisk denotes significant differences in survival between treated and untreated cultures as determined by Student's t-test ($p < 0.05$). Panel (c): Replicate cultures of C6 rat glioma cells and primary embryonic rat brain cultures were established and treated with various concentrations of acetaminophen for 2 days. Relative cell numbers were obtained, as above. Solid line: primary brain cultures. Dashed line: C6 cultures. Panel (d): Cells were cultured and treated as described for panel (c), except that cells were counted after 5 days.

relative reduction in cell number resulted in no significant effects on cell number in primary brain cultures. However, since the rate of cell division in non-tumor cell cultures was generally slower, the same type of comparison was made after 2 and 5 days of treatment using C6 glioma cells and primary cultures from embryonic rat brain. Results are illustrated in panels (c) and (d) of this figure. Comparison of dashed lines in both panels indicates that cell death in the tumor cell cultures was both dose dependent and progressive. In contrast, normal cells remained resistant to the same concentrations of acetaminophen (solid lines).

Acetaminophen increases radiation sensitivity in SNB-19 glioma cells

In order to determine whether acetaminophen could increase the radiosensitivity of SNB-19 human glioma cells, clonogenic assays were performed. Results are illustrated in Figure 7. Under these conditions acetaminophen was not toxic, resulting in no differences in clone numbers after two weeks in the absence of radiation, measured at $92 \pm 7\%$ of cells plated for control cultures, and $85 \pm 4\%$ of cells plated for acetaminophen-treated cultures. Since acetaminophen could significantly increase mitotic cell numbers (Figure 3B) after 3.5 h of treatment, we selected the same interval between drug treatment and cell radiation. Results demonstrate significant reductions in survival fractions with acetaminophen treatment for radiation doses of 2, 4, 5 and 6 Gy, with decreases on the order of 26%, 30%, 40%, and 40% for these doses of radiation, respectively.

Discussion

In this study we have demonstrated that acetylsalicylate, acetaminophen, and ibuprofen all significantly

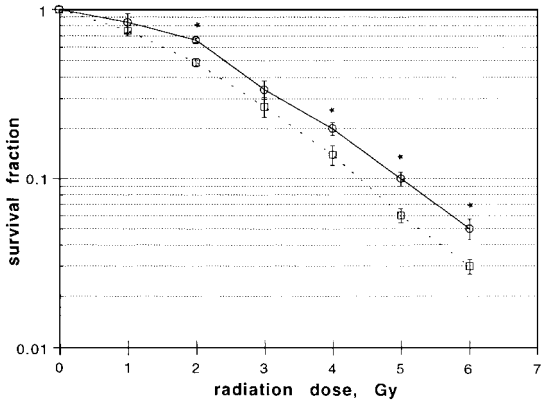

Figure 7. Acetaminophen increases radiosensitivity of SNB-19 glioma cells in culture. Triplicate cultures of SNW-19 cells were treated with 0.5 mM acetaminophen (or vehicle) for 4 h, after which cultures were irradiated at various doses. Solid line: control survival curve. Dotted line: survival curve in the presence of acetaminophen. Asterisks denote significant differences in the survival fraction between treated and control cultures at a given dose, as determined by Student's t-test ($p < 0.05$).

reduce the numbers of glioma cells in culture at low millimolar doses over the course of several days. This concentration range is witnin the therapeutic range for acetylsalicylate (150–300 μg/ml, or 0.5–1 mM in plasma) but above the human therapeutic range for acetaminophen (10–20 μg/ml, or 0.06–0.13 mM in plasma). However, the toxic serum concentration reported for acetaminophen (300 μg/ml, or 2 mM) is much higher than the dose required to produce significant toxic effects in our experiments (Figure 1). The toxicity range for ibuprofen is less clear, although anecdotal cases of severe adverse effects in children have been reported [28].

We are the first to demonstrate that acetaminophen produced significant reductions in both human and rat glioma cell numbers in culture, with the dose response curve falling between those for acetylsalicylate and ibuprofen. Higher concentrations of acetaminophen were more effective after longer incubation times (Figure 6c,d), and laboratory analysis of the acetaminophen concentration in culture supernatants from our experiments, measured in the range of 140–148 μg/ml, gave no indication of changes in concentration over 4 days. Although an acidification of the medium was apparent in cultures treated with high doses of acetylsalicylate and ibuprofen, it probably did not account for the toxicity, since acetylsalicylate is the strongest acid, but had the least potent toxic effect. Each acid was titrated to determine its apparent pK (−log dissociation constant), as calculated by the Henderson–Hasselbach equation, and found to be 3.9 for acetylsalicylate, 9.0 for acetaminophen, and 4.6 for ibuprofen. Since the rank order of pK's does not correlate with the relative toxicities of these compounds, they are probably acting via another pathway.

Ibuprofen was measured to produce the most potent effects; a 3.3 mM dose was able to eradicate all cells from the culture dish, confirming that its actions were toxic, rather than proliferation-inhibiting, which would also result in no increases in cell numbers over time. This result is supported by previously published results that systemic administration of ibuprofen was able to decrease the size of C6 glioma spheroid cells implanted to rat striatum in an *in vivo* brain tumor model [17]. However, no conclusions could be made about whether the drug was acting directly on glioma cells, or on the host vasculature and/or immune response. Ibuprofen, as are the other compounds, is a potent inhibitor of prostaglandins, which are known to mediate angiogenesis [18] and modulate the immune response by inhibiting the release of cytokines in blood [29]. In our study we have demonstrated a direct toxic effect of this compound on tumor cells themselves.

In addition, our results are consistent with a previous report that acetylsalicylate was toxic for the rat glioma cell line RG2 both *in vivo* and *in vitro* with a similar dose, in that case, 2 mM [20]. Detailed dose response and time course experiments demonstrated that these parameters varied similarly to those of ibuprofen, and that acetylsalicylate effectively killed all of the cells in culture at higher doses (in this case 10 mM). However, with 1 mM acetylsalicylate, both SNB-19 and C6 cell numbers remained the same for 2 days, after which time they increased (Figure 2). Since acetylsalicylate degrades to salicylic acid and acetate, it is possible that acetylsalicylate loses activity in culture, and cells are then able to recover.

Acetylsalicylate, acetaminophen, and ibuprofen display differential effects on the cell cycle

During normal development, cells exhibit the capacity to rapidly divide, generating significant numbers of cells which differentiate into various phenotypes and perform various functions. Concomitant with this process is another process called programmed cell death, or apoptosis, which leads to cell death. Recently, it has been found that mitosis and apoptosis require the expression of many common genes [30,31]. Thus, an

attractive hypothesis is that since tumor cells recapture their mitotic status after oncogenic transformation, they may be shifted into the apoptotic mode more readily than normal cells. In order to further examine the mechanism by which acetylsalicylate, acetaminophen, and ibuprofen reduce cell numbers, their effects on the mitotic status of a population of glioma cells in culture were investigated. These results also allowed us to distinguish between the inhibition of proliferation and actual cell killing. Our results demonstrate that although there was a relative decrease in cell number over time with respect to the exponential growth of control cultures (Figure 2), acetaminophen and ibuprofen, but not acetylsalicylate produced a transient increase in cell number after 3.5 h (Figure 3A). Interestingly, using the MC6 antibody to identify mitotic cells, as previously reported and characterized by Vincent and colleagues [26], we obtained different results with acetaminophen and ibuprofen. While acetaminophen and ibuprofen transiently increased cell number, only acetaminophen increased the number of mitotic cells over the same time course. Finally, BrdU labeling followed by immunocytochemistry for BrdU-labeled DNA revealed that both acetaminophen and ibuprofen significantly decreased the number of cells that synthesized DNA during this period. Schiff and colleagues [32] have examined the effects of NSAIDs on the cell cycle in HT-29 colon adenocarcinoma cells *in vitro*. Their study demonstrated an increased proportion of cells in G0/G1, the cell cycle phase following mitosis, resulting in a reduced percentage of cells at the G2/M cell cycle checkpoint, and a decreased proportion in S-phase in response to acetylsalicylate. In contrast, we observed a trend towards an increase in S-phase cells with acetylsalicylate, possibly due to: (1) differences in cell cycle kinetics for these cell lines, or (2) alternate mechanisms determined specifically by each cell type. Interestingly, similar experiments performed after 24 and 48 h of drug treatments failed to demonstrate significant increases in M-phase cells (data not shown), supporting the idea that cells died a 'mitotic' cell death. In order to reconcile these observations, more detailed analyses of the kinetics of the cell cycle and the proportions of cells in each phase are required, using similar methods as those published, such as propidium iodide labeling followed by fluorescence-activated cell sorting, in order to directly compare results.

Observations using light microscopy on fixed preparations of cells treated for several days with acetylsalicylate, acetaminophen, or ibuprofen revealed further distinctions in the manner in which these drugs act in culture. As illustrated in Figure 4, each drug produced its own distinctive morphological response when the analysis was made at the same point in time. Acetylsalicylate treatment resulted in more subtle morphological changes (membrane ruffling). Acetaminophen produced a more drastic change in cell shape; cells which normally grow in a symmetrical fashion, similar to cultured primary type I astrocytes [33], assumed spindle shapes. In addition, condensed, phase-bright material accumulated in the culture dish. The effects of ibuprofen were the most dramatic, with more abundant accumulations of debris, and the condensation of cell cytoplasm and nuclei, accompanied by the elaboration of microspikes of cytoplasm, reminiscent of cells dying by apoptosis.

The significance of the differences in relative efficacies, kinetics, cell cycle alterations, and morphologies produced by these drugs is not known at this time, but taken together, these differences have several interpretations. One possibility is that each drug has a distinct interaction with the cells, producing unique effects based on different mechanisms of action. The second possibility is that these drugs act by a common mechanism, but since they have varying potencies, the magnitude and rate of the cellular responses are different. Therefore, the morphological changes seen with these drugs are reflections of the time course of the response to each drug. For example, the response to ibuprofen was the fastest, producing the most devastating effects on cells; the response to acetylsalicylate was the least drastic. Additionally, acetylsalicylate was not found to have an effect on total cell number, mitotic cell number, or DNA synthesis after 3.5 h of culture, whereas the other two drugs had significant effects. The difference between them is the number of cells found to be dividing after 3.5 h. Since ibuprofen decreased cell numbers at the fastest rate, it is possible that the transient increase in cell numbers took place at an accelerated rate; after 3.5 h there were more total cells, but less mitotic ones, because they had already either completed mitosis or died.

Several mechanisms of toxicity for acetylsalicylate, acetaminophen, and ibuprofen are possible

Many compounds, including acetylsalicylate, acetaminophen, and ibuprofen, inhibit prostaglandin formation by inhibiting a mixed function enzyme,

cyclooxygenase [34–37]. Two forms of this enzyme have been identified and cloned: COX-1 and COX-2 [38]. In a study by Vane and Botting which examined and ranked the relative potencies of NSAIDs and related compounds on the inhibition of COX-1 and COX-2, the IC_{50}'s (in μM) for these drugs respectively were: 1.67 and 278 for acetylsalicylate, 17.9 and 133 for acetaminophen, and 4.85 and 72.8 for ibuprofen, with ibuprofen requiring the lowest dose for 50% inhibition of COX-2 [39]. Similarly, doses required to reduce glioma cell number by 50% after two days of incubation for acetylsalicylate, acetaminophen, and ibuprofen are 3, 1, and 0.7 mM, respectively. Comparing the potencies of acetylsalicylate, acetaminophen, and ibuprofen with respect to their COX-2 inhibition, the ratio of COX-2 IC_{50}'s for acetylsalicylate and ibuprofen compared with acetaminophen are 2.1 and 0.55, respectively. These values are similar to the ratios of the concentrations of each drug required to produce a 50% reduction in cell number with respect to acetaminophen (3.0 and 0.7). The hypothesis that NSAIDs directly act on tumor cell growth by the inhibition of prostaglandin synthesis has been disputed [40], but a recent report demonstrating NSAID-induced apoptosis in colorectal cancer cell lines supports this hypothesis. In their study, Chan and colleagues determined that it was not the decrease in prostaglandin formation after indomethacin or sulindac that induced cell death, but rather the increase in the concentration of arachidonic acid, a precursor of prostaglandins, which induced ceramide formation from sphingomyelin. Ceramide-induced apoptosis ensued [21]. However, this pathway may not be the mechanism of toxicity in tumors of glial origin, as indomethacin was shown to have no toxic effects on 9L glioma cells in culture [41]. Regardless of the mechanism by which these drugs act directly, the inhibition of prostaglandin synthesis would also decrease tumor size indirectly by inhibiting angiogenesis, a process in which prostaglandins play a major role [18].

In the liver and kidney, two organs of drug catabolism, acetaminophen is arylated and oxidized by mixed-function oxidases, such as cytochrome P450, to form N-acetyl-p-benzoquinone imine (NAPQI). This highly reactive compound produces cell damage and leads to cell death [42]. NAPQI is normally detoxified by reduced glutathione, but high doses of acetaminophen will exceed glutathione availability, which may influence apoptosis and necrosis [43]. In PC12 cells engineered to express cytochrome P450 E1, acetaminophen produced both growth retardation and cell death [44]. Although this mechanism could theoretically result in cell death in our system, we believe that it is not the major determinant of acetaminophen-induced toxicity. Firstly, the rank order of potencies of acetylsalicylate, acetaminophen, and ibuprofen directly correlate with their ability to inhibit COX-2, as discussed above. Secondly, although ethanol has been demonstrated to both potentiate and inhibit cytochrome P450 [45,46], concentrations of ethanol ranging from 0.5% to 1.0% did not affect acetaminophen toxicity or radiosensitivity. Furthermore, substitution of ethanol with dimethyl sulfoxide (DMSO) did not significantly change the magnitude of acetaminophen toxicity. Finally, glutathione levels of acetaminophen-treated and control cultures did not differ significantly from 2 to 48 h after treatment (data not shown).

As discussed earlier, changes in extracellular pH are probably not responsible for the toxicity observed in our experiments. All compounds tested were weak acids, but it was acetylsalicylate, the least potent compound of the three, which had the lowest pK, 3.8. However, acetylsalicylate may result in alterations of intracellular pH indirectly. Aas and colleagues have demonstrated that acetylsalicylate can inhibit the sodium-dependent bicarbonate ion pump [20], resulting in decreases in cellular pH. At this time it is not known whether acetaminophen or ibuprofen have similar effects.

Acetaminophen is selectively toxic for dividing cells and has potential as a therapeutic agent for brain tumors

Acetaminophen has been the least studied of the over-the-counter analgesics with respect to cancer because acetaminophen is only weakly anti-inflammatory, and the previously held belief was that the mechanism of action of NSAIDs was through their anti-inflammatory actions [47]. However, several published studies demonstrating that these drugs may act through alternative mechanisms [48] have led to new interest in the actions of acetaminophen on cancer risk in humans [49].

We have examined the effects of acetaminophen in more detail with respect to the mechanism of the direct reduction in cell numbers *in vitro*. In order to make the distinction between apoptosis, also called 'programmed', or 'developmental' cell death, and necrosis, often considered a reaction to a substantive

pathologic insult, we have examined cultures treated with acetaminophen for morphological and biochemical evidence of these processes. During apoptotic cell death, there is chromatin margination, nuclear condensation, and shrinkage followed by blebbing of cytoplasm and disintegration into apoptotic bodies. Necrosis is characterized by swelling and lysis of organelles. Some of the features of apoptotic cell death, such as nuclear condensation and shrinkage, have been observed in our cultures of SNB-19 cells treated with ibuprofen (Figure 4d). TUNEL, analysis by immunocytochemical detection of digoxigenylated end labeled DNA fragments in acetaminophen-treated cultures revealed an up to 8-fold increase in the relative numbers of apoptotic cells. However, this represented a small fraction of the total cell number observed in the culture dish; only up to 0.1% of cells were stained with our methods. Therefore it is possible that the majority of the cells could be dying by another mechanism, or that the remaining apoptotic cells represented the majority of dying cells which were washed off the culture dish while being processed for labeling. Results from studies on other cell types suggest that the kinetics of apoptosis in response to acetaminophen may be rapid. In liver cells treated with acetaminophen, nuclear condensation increased significantly after 2 h [50], with evidence of DNA laddering within 2–24 h. Future experiments conducted over shorter incubation times with various fixation methods will determine whether glioma cells die by necrosis or apoptosis (or both) when exposed to acetaminophen.

GBM is a highly lethal brain cancer, accounting for a large percentage of primary brain tumors in adults [51]. Although radiation therapy (RT) can extend patient survival [52], the sensitivity of the surrounding functional brain limits the safe dose of RT to a level well below that required for complete tumor eradication. It is generally recognized that the damage caused to cells by ionizing radiation is the result of induction of lesions in the nuclear DNA. Cells undergoing mitosis are more vulnerable to radiation [53]. Conventional RT induces death predominantly in cells which are cycling at the time radiation is delivered. Since GBM tumors have a low mitotic index [54], increasing the number of cells undergoing mitosis will increase the fragility of these cells when exposed to radiation. In this study we have demonstrated by an *in vitro* clonogenic assay that acetaminophen can increase the radiosensitvity of human glioma cells up to 40% (Figure 7). We have also demonstrated that when compared directly with human or rodent brain cells in culture, glioma cells are more vulnerable to the toxic effects of acetaminophen (Figure 6). These results support the hypothesis that acetaminophen can influence the mitotic index of cells in culture. The selectivity of acetaminophen for glioma cells and its ability to increase radiosensitivity in culture suggest that this strategy may have clinical application. Although high doses of acetaminophen are toxic to other organs such as the liver and the kidney [55], N-acetyl-cysteine has been used clinically to protect the body from these actions [56]. Furthermore, alternative strategies for delivery of acetaminophen, such as stereotaxic injection, could be considered.

In conclusion, results from this study demonstrate that acetylsalicylate, acetaminophen and ibuprofen are directly toxic to both human and rodent glioma cell lines. The fact that these compounds produce similar effects on both human and animal cells leads to the suggestion that these compounds will be toxic to other glioma cells and cell lines as well. Evidence indicating differential sensitivity of tumor cells compared with non-tumor cells to acetaminophen and the ability of acetaminophen to increase radiosensitivity may lead to new clinical strategies in the treatment of brain tumors. Planned experiments will further examine the possible mechanisms of action for these drugs, because better understanding of how these drugs act may lead to improved treatment strategies.

Acknowledgements

This work was supported in part by a pilot research grant from the Resnick Gerontology Center at the Montefiore Medical Center. The authors would like to thank the staff in the Departments of Radiation Oncology and Hematology at the Montefiore Medical Center for assisting in the irradiation of cell cultures, Dr. Peter Davies in the Department of Pathology at the Albert Einstein College of Medicine for critical comments on the manuscript, and Ms. Alexandra Neuhaus-Follini for her editorial assistance.

References

1. Karim SM, Rao B: Prostaglandins and tumors. Adv Prostaglandin Res 2: 303–325, 1976
2. Thun MJ: Aspirin, NSAIDs, and digestive tract cancers. Cancer Metastas Rev 13: 269–277, 1994

3. Thun M, Namboodiri M, Heath C: Aspirin use and reduced risk of fatal colon cancer. N Engl J Med 325: 1593–1596, 1991
4. Logan RFA, Little J, Hawtin PG, Hardcastle JD: Effect of aspirin and non-steroidal anti-inflammatory drugs on colorectal adenomas: case-control study of subjects participating in the Nottingham faecal occult blood screening programme. Br Med J 307: 285–289, 1993
5. Peleg II, Maibach HT, Brown SH, Wilcox CM: Aspirin and nonsteroidal anti-inflammatory drug use and the risk of subsequent colorectal cancer. Arch Intern Med 154: 394–399, 1994
6. Cramer DW, Harlow BL, Titus-Emstoff L, Bohike K, Welch WR, Greenberg ER: Over-the-counter analgesics and risk of ovarian cancer. Lancet 351: 104–107, 1998
7. Kobrinsky NL, Hartfield D, Horner H, Maksymiuk A, Minuk GY, White DF, Feldstein TJ: Treatment of advanced malignancies with high-dose acetaminophen and *N*-acetylcysteine rescue. Cancer Invest 14: 202–210, 1996
8. Ross RK, Paganini-Hill A, Landolph J, Gerkins V, Henderson BE: Analgesics, cigarette smoking, and other risk factors for cancer of the renal pelvis and ureter. Cancer Res 49: 1045–1048, 1989
9. McCredie M, Stewart JH, Day NE: Different roles for phenacetin and paracetamol in cancer of the kidney and renal pelvis. Int J Cancer 53: 245–249, 1993
10. Linet MS, Chow WH, McLaughlin JK, Wacholder S, Yu MC, Schoenberg JB, Lynch C, Fraumeni Jr JF: Analgesics and cancers of the renal pelvis and ureter. Int J Cancer 62: 1–18, 1995
11. Grodstein F, Martinez E, Platz EA, Giovannucci E, Colditz GA, Kautzky M, Fuchs C, Stampfer MJ: Postmenopausal hormone use and risk for colorectal cancer and adenoma. Ann Intern Med 128: 705–712, 1998
12. Hial V, Horakova Z, Shaff RE, Beaven MA: Alteration of tumor growth by aspirin and indomethacin: studies with two transplantable tumors in mouse. Eur J Pharmacol 37: 367–376, 1976
13. Lynch NR, Castes M, Astoin M, Salomon J: Mechanism of inhibition of tumour growth by aspirin and indomethacin. Br J Cancer 38: 503–512, 1978
14. Narisawa T, Satoh M, Sano M, Takahashi T: Inhibition of initiation and promotion bv N methylnitrosourea-induced colon carcinogenesis in rats by non-steroid anti-inflammatory agent indomethacin. Carcinogenesis 4: 1225–1227, 1983
15. Fulton AM: Effects of indomethacin on the growth of cultured mammary tumors. Int J Cancer 33: 375–379, 1984
16. Kort WJ, Huslman LOM, van Schalkwijk WP, Weijma IM, Zondervan PE, Westbroek DL: Reductive effect of aspirin treatment on primary tumor growth and metastasis of implanted fibrosarcoma in rats. JNCI 76: 711–720, 1986
17. Farrell CL, Megyesi J, Del Maestro RF: Effect of ibuprofen on tumor growth in the C6 spheroid implantation glioma model. J Neurosurg 68: 925–930, 1988
18. Ziche M, Jones J, Gullino PM: Role of prostaglandin E_1 and copper in angiogenesis. JNCI 69: 475–482, 1982
19. Patierno SR, Lehman NL, Henderson BE, Landolph JR: Study of the ability of phenacetin, acetaminophen, and aspirin to induce cytotoxicity, mutation, and morphological transformation in C3H/ 10T1/2 clone 8 mouse embryo cells. Cancer Res 49: 1038–1044, 1989
20. Aas AT, Tønnessen TI, Brun A, Salford LG: Growth inhibition of rat glioma cells *in vitro* and *in vivo* by aspirin. J Neuro-Oncol 24: 171–180, 1995
21. Chan TA, Morin PT, Vogelstein B, Kinzler KW: Mechanisms underlying nonsteroidal antiinflammatory drug-mediated apoptosis. Proc Natl Acad Sci USA 95: 681–686, 1998
22. Gross JL, Behrens DL, Mullins DE, Kornblith PL, Dexter DL: Plasminogen activator and inhibitor activity in human glioma cells and modulation by sodium butyrate. Cancer Res 48: 291–296, 1988
23. Benda P, Lightbody J, Sato G, Levine L, Sweet W: Differentiated rat glial cell strain in tissue culture. Science 161: 370–371, 1968
24. Gavrieli Y, Sherman Y, Ben-Sasson SA: Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. J Cell Biol 119: 493–501, 1992
25. Puck TT. Marcus PI: Action of X-rays on mammalian cells. J Exp Med 103: 653–666, 1956
26. Vincent I, Rosado M, Davies P: Mitotic mechanisms in Alzheimer's disease? J Cell Biol 132: 413–425, 1996
27. Shen W, Kamendulis LM, Ray SD, Corcoran GB: Acetaminophen-induced cytotoxicity in cultured mouse hepatocytes: Effects of Ca^{2+}-endonuclease, DNA repair, and glutathione depletion inhibitors on DNA fragmentation and cell death. Toxicol Appl Pharmacol 112: 32–40, 1992
28. McEvoy GK: American Hospital Formulary Service: Drug Information. American Society of Health-System Pharmacists, 1997
29. Endres S, Whitaker RE, Ghorbani R, Meydani SN: Oral aspirin and ibuprofen increase cytokine-induced synthesis of IL-1 beta of tumour necrosis factor-alpha *ex vivo*. Immunology 87: 264–270, 1996
30. Steller H: Mechanisms and genes of cellular suicide Science 267: 1445–1449, 1995
31. Ucker D: Death by suicide: one way to go in mammalian cellular development. New Biol 3: 413–425, 1991
32. Schiff SJ, Koutsos MI, Qiao L, Rigas B: Nonsteroidal antiinflammatory drugs inhibit the proliferation of colon adenocarcinoma cells: effects on cell cycle and apoptosis. Exp Cell Res 222: 179–188, 1996
33. Raff MC, Mirsky R, Fields KL, Lisak RP, Dorfman SH, Siberberg DH, Gregson NA, Liebowitz S, Kennedy MC: Cell type specific markers for distinguishing and studying neurons and the major classes of glial cells in culture. Brain Res 174: 283–308, 1979
34. Vane J: Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nature 231: 232–235, 1971
35. Flower RJ: Drugs which inhibit prostaglandin biosynthesis. Pharmacol Rev 26: 33–67, 1974
36. Lewis GP: Prostaglandins in inflammation. J Reticuloendothel Soc 22: 389–402, 1977
37. Shen TY: Prostaglandin synthetase inhibitors. In: Vane JR, Ferreirs SH (eds) Antiinflammatory Drugs. Springer-Verlag, Berlin, 1979, pp 305–347

38. Feng L, Sun W, Xia Y, Tang WW, Chanmugam P, Soyoola F, Wilson CB, Hwang D: Cloning two isoforms of rat cyclooxygenase: differential regulation of their expression. Arch Biochem Biophys 307: 361–368, 1993
39. Vane JR, Botting RM: New insights into the mode of action of anti-inflammatory drugs. Inflamm Res 44: 1–10, 1995
40. Hanif R, Pittas A, Feng Y, Koutsos MI, Qiao L, Staiano-Coico L, Shiff SI, Rigas B: Effects of non-steroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. Biochem Pharmacol 52: 237–245, 1996
41. Karuri AR, Dobrowsky F, Tannock IF: Selective cellular acidification and toxicity of weak organic acids in an acidic microenvironment. Br J Cancer 68: 1080-1087, 1993
42. Danlin DC, Miwa GT, Lu AY, Nelson EC: N-acetyl-p-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. Proc Natl Acad Sci USA 81: 1327–1331, 1984
43. Fernandes RS, Cotter, TG: Apoptosis or necrosis: Intracellular levels of glutathione influence mode of cell death. Biochem Pharmacol 48: 675–681, 1994
44. Holownia A, Mapoles J, Menez JF: Acetaminophen metabolism and cytotoxicity in PC12 cells transfected with cytochrome P4502E1. J Mol Med 75: 522–527, 1997
45. Roberts BJ, Song B-Y, Soh Y, Park SS, Shoaf SE: Ethanol induces CYP2E1 by protein stabilization. J Biol Chem 270: 29632–29635, 1995
46. Sato C, Lieber CS: Mechanism of the preventive effect of ethanol on acetaminophen-induced hepatotoxicity. J Pharmacol Exp Ther 218: 805–810, 1981
47. Woodbury DM, Fingl E: Analgesic-antipyretics, anti-inflammatory agents, and drugs employed in the treatment of gout. In: Goodman LS, Gilman A (eds) The Pharmacological Basis of Therapeutics. Macmillan Company, New York, 1975, pp 329–334
48. Grilli M, Pizzi M, Memo M, Spano PF: Neuroprotection by aspirin and sodium salicylate through blockade of NF-κB. Science 74: 1383–1385, 1996
49. Cramer DW, Harlow BL, Titus-Emstoff L, Bohike K, Welch WR, Greenberg ER: Over-the-counter analgesics and risk of ovarian cancer. Lancet 351: 104–107, 1998
50. Ray SD, Mumaw VR, Raje RR, Fariss MW: Protection of acetaminophen-induced hepatocellular apoptosis and necrosis by cholesteryl hemisuccinate pretreatment. J Pharmacol Exp Ther 279: 1470–1483, 1996
51. Kornblith PL, Walker MD, Cassady JR (eds): Neurologic Oncology. JB Lippincott, Philadelphia, 1987, pp 35–48
52. Shapiro WR, Green SB, Burger PC, Mahaley Jr MS, Selker RG, VanGilder JC, Robertson JT, Ransohoff J, Mealey Jr J, Strike TA, Pistenmaa DA: Randomized trial of three chemotherapy regimens and two radiotherapy regimens in postoperative treatment of malignant glioma. J Neurosurg 71: 1–9, 1989
53. Sinclair WK: Cyclic X-ray responses in mammalian cells *in vitro*. Radiat Res 33: 620–643, 1968
54. Yoshii Y, Maki Y, Tsuboi K, Tomono Y, Nakagawa K, Hoshino T: Estimation of growth fraction with bromodeoxyuridine in human central nervous system tumors. J Neurosurg 65: 659–663, 1986
55. Jollow DJ, Mitchell JR, Potter WZ, Davies DC, Gillette JR, Brodie BB: Acetaminophen-induced hepatic necrosis. II. Role of covalent binding *in vivo*. J Pharmacol Exp Ther 187: 195–202, 1973
56. Prescott LF, Park J, Ballentyne A, Park J, Anriarnssens P: Treatment of paracetamol (acetaminophen) poisoning with N-acetyl-cysteine. Lancet 2(8035): 432–434, 1977

Address for offprints: Diana Casper, Department of Neurological Surgery, Moses Building, Room 314, Montefiore Medical Center, 111 East 210th Street, The Bronx, New York 10467, USA; Tel.: 718-920-4064 ; Fax: 718-653-3284