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Early changes in metabolism of leukemic cell lines upon induction of apoptosis by cytotoxic drugs

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Abstract

We evaluated real-time changes in extracellular acidification rates of human U937 and K562 leukemic cells treated with camptothecin or taxol. U937 cells treated with camptothecin or taxol for 30–60 min showed a continuous, irreversible decrease in extracellular acidification rate that was sensitive to amiloride. In contrast, U937 cells exposed to sodium azide showed an immediate, steep decrease in extracellular acidification rate that was reversible upon azide withdrawal. K562 cells required a >20-fold higher dose of camptothecin to promote similar changes in the extracellular acidification rate, with a corresponding resistance in their susceptibility to camptothecin- or taxol-induced apoptosis. The data show that irreversible commitment to apoptosis is associated with rapid metabolic changes that are reflected by decreased extracellular acidification rate and regulated by the Na^+/H^+ antiporter. Moreover, detection of extracellular acidification rate changes was not restricted to a particular cell type or apoptosis pathway, making this a potentially useful tool to screen compounds for pro-apoptotic activity.
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Keywords: Apoptosis; Microphysiometry; Extracellular acidification rate; Na^+/H^+ antiporter; Leukemic cell line

1. Introduction

Cell death is an important physiological process that can be triggered by physiological or nonphysiological stimuli. Apoptosis is the most common form of physiological cell death and occurs during embryonic development, tissue remodeling, immune regulation, and tumor regression (Schulze-Osthoff et al., 1998). In contrast, necrosis results from stimuli that lead to "nonphysiological" cell death during traumatic injury or exposure to high concentrations of noxious agents (Raffray and Cohen, 1997). Physiological cell death processes can be activated by a variety of signals, such as growth factor deprivation (Modiano et al., 1999; Pittman et al., 1993) and disturbance in intracellular pH homeostasis (Chinnaiyan et al., 1996). Cells regulate their pH through a variety of ion transport mechanisms (Madshus, 1988). The Na^+/H^+ antiporter, which is driven by the inward-directed Na^+ gradient, is a major H^+-export mechanism. Intracellular acidification has been shown to be an early event in apoptosis (Gottlieb et al., 1996; Meisenholder et al., 1996).

The Cytosensor™ microphysiometer (Molecular Devices, Sunnyvale, CA) is a unique device capable of real-time measurements of changes in cell metabolism. The principle of operation of this instrument is based on the presence of a light-addressable potentiometric sensor (LAPS), which consists of lightly doped silicon with a thin silicon nitride insulator that contacts an aqueous solution. The LAPS resides within a cell culture chamber that has continuous flow of media. Alterations in the electric field (voltage gradient) in the silicon chamber are proportional to changes in surface potential and therefore to changes in the solution pH. The pH dependence of the surface potential is Nernstian, ~59 mV/pH unit at room temperature. A rate of

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change of sensor output voltage of 1 μV/s corresponds closely to an extracellular acidification rate of 1×10^{-3} pH unit/min.

The extracellular acidification rate is dependent on the export of intracellular protons from cells in the tissue culture chamber (Owicki and Parce, 1992) and therefore on the activity of energy-producing metabolic pathways (glycolysis and aerobic respiration). Both metabolic pathways produce protons through formation of the acidic byproducts (lactic acid and CO_2, respectively). Decreased metabolic activity (reduced extracellular acidification rate) could be due to (i) suppression of cellular metabolism; (ii) conversion from glycolytic to aerobic metabolism resulting in fewer protons per ATP molecule; (iii) cytotoxicity (cell death), or combinations thereof.

Alterations in mitochondrial function during the execution of apoptosis have been characterized in detail, but early changes in cellular metabolism in cells committed to die are less well understood. In the present study, continuous monitoring of extracellular acidification rate before, during, and after removal of camptothecin or taxol, two agents that can induce apoptosis, was used to evaluate the effects of these compounds on metabolism in leukemic cell lines. The data show that the irreversible commitment to apoptosis is associated with early changes in cellular metabolism as reflected by a continuous decrease in extracellular acidification rate. These changes in extracellular acidification rate were sensitive to amiloride, suggesting that they were mediated by the Na^+/H^+ antiporter. The measurement of extracellular acidification rate is noninvasive, and thus, there is no requirement for the introduction of fluorescent dyes or colorimetric reporter probes. In addition, the detection of these extracellular acidification rate changes is not restricted to a particular cell type, making the assessment of extracellular acidification rate a potentially useful tool to screen novel compounds for apoptotic activity.

2. Materials and methods

2.1. Cell lines and reagents

The U937 (human myelomonocytic leukemia) and K562 (human erythroleukemia) cell lines were obtained from the American Type Culture Collection (Rockville, MD). RPMI-1640 medium and 100-X solutions of L-glutamine, HEPES buffer, and sodium pyruvate were obtained from Gibco BRL (Life Technologies, Bethesda, MA). Fetal bovine serum was obtained from Hyclone (Logan, UT). Camptothecin, taxol, and sodium azide were purchased from Sigma (St. Louis, MO). 5-*N*-methyl-*N*-isobutyl amiloride was obtained from Calbiochem (La Jolla, CA). Camptothecin was dissolved in 0.5 M NaOH to a concentration of 150 mM (50 mg/ml); taxol was dissolved in dimethyl-sulphoxide (DMSO) to a concentration of 10 mM; sodium azide and amiloride were dissolved in "running media" (bicarbonate-free Dulbecco's Modified Eagle Media (DMEM) supplemented with 0.5 mg/ml bovine serum albumin). All stock solutions were stored at −20 °C. Compounds were diluted to working concentrations in "running media" immediately prior to use and the pH was adjusted to 7.4. Equivalent amounts of the vehicles used to dissolve the camptothecin (0.5 M NaOH) and taxol (DMSO) were added to control conditions; neither affected apoptosis or pH measurements. Trans-well™ cell capsules (tissue culture treated) were obtained from Costar (Cambridge, MA). Cytosensor™ capsule inserts, spacer gasket, and low-melting-point agarose were obtained from Molecular Devices.

2.2. Cell culture

Cells were grown in suspension culture in complete media (RPMI 1640 supplemented with 10% fetal bovine serum, sodium pyruvate, HEPES buffer, and 2 mM L-glutamine) at 37 °C in a humidified 5% CO_2 atmosphere. Cell cultures were maintained in exponential growth by media replacement every 2 to 3 days or subculture at a 1:3 dilution. Cells were provided fresh media the day before they were used for experimentation.

2.3. Evaluation of apoptosis

Apoptotic cell death was evaluated by terminal deoxytransferase (TdT) labeling of DNA fragments using the In Situ Cell Death Detection Kit-Fluorescein (Boehringer Mannheim, Indianapolis, IN) as per the manufacturer's instructions. Camptothecin and taxol were diluted to working concentrations in "incubation media" immediately prior to use, and the pH was adjusted to 7.4. Equivalent amounts of the vehicles used to dissolve the camptothecin (0.5 M NaOH) and taxol (DMSO) were added to control conditions. Cells were analyzed flow cytometrically with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA).

2.4. Microphysiometry

Single cell suspensions were mixed with low-temperature-melting agarose at a ratio of 3:1 (v/v). Ten microliters of the agarose–cell mixture containing 5×10^4 U937 cells or 1×10^5 K562 cells was immediately spotted onto the membrane of a Cytosensor™ cell capsule. Cells were cultured in the Cytosensor™ cell capsule cup in complete media for 10 to 12 h before replacing it with serum-free media to reduce basal metabolic rate (2–3 h). Prior to measurements, the media in the capsule cup was replaced with "running media." The Cytosensor™ chamber was maintained at 37 °C. The flow of running media through the sensor chamber assembly was initially maintained at 100 μl/min, and the voltage signal, which is proportional to extracellular pH, was measured and recorded. Acidification rates were determined by measuring the rate of pH change

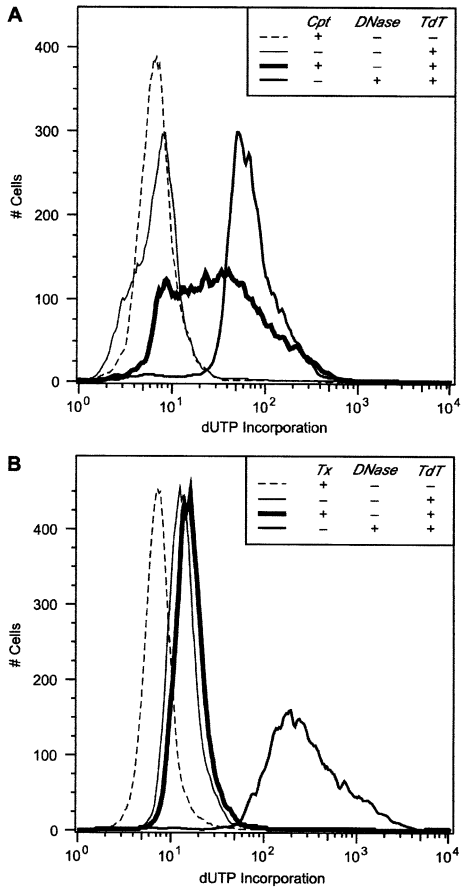

Fig. 1. Susceptibility of camptothecin-treated U937 cells and resistance of taxol-treated K562 cells to apoptosis. (A) U937 cells (1×10^6) were cultured in the presence or absence of camptothecin (Cpt, 3 μM) for 6 h as indicated. Control cells were treated with an equivalent amount of vehicle (0.5 M NaOH added to achieve a final concentration of 50 μM NaOH). At the end of the culture period, cells were fixed and permeabilized, and DNA fragmentation was examined by TUNEL. (B) K562 cells (1×10^6) were cultured in the presence or absence of taxol (Tx, 1 μM) for 6 h. Control cells were treated with an equivalent amount of vehicle (DMSO added at a 1:10,000 proportion of the final volume). At the end of the culture period, cells were fixed and permeabilized, and DNA fragmentation was examined by TUNEL. For negative controls (dashed line), TdT was omitted from the labeling step in the TUNEL assay using cells treated with camptothecin (A) or with taxol (B). For positive controls, fixed and permeabilized cells that did not receive camptothecin (A) or taxol (B) were treated with DNase (2.5 μg) for 12 min prior to the addition of TdT.

during periodic 30 s halts in the flow of running media through the flow chamber to permit accumulation of cellular metabolites (e.g., lactic acid). Acidification rates (in the absence of any stimulation) were monitored until a stable baseline was achieved (usually within 1–2 h). Once the resting baseline was stable, cells were treated with camptothecin, taxol, or sodium azide at the indicated concentrations. The concentrations used for the microphysiometry experiments were higher than those used for the static experiments assessing DNA fragmentation to account for the continuous flow rate that washed out the compound and also for the reduced exposure time. For some experiments, cells were exposed to amiloride (10 μM) prior to treatment with camptothecin. Extracellular acidification rate was measured for 5 to 8 h and normalized to reflect percent change, where the μV/s measurement prior to treatment was considered 100%; changes are shown as a function of real time. The normalization permits direct comparisons among experiments where cells had different initial extracellular acidification rate levels and accounts for possible variations in the number of cells residing above the active sensing region of the light-addressable potentiometric sensor chip in the chamber.

3. Results

We evaluated the capacity of camptothecin and taxol to promote apoptosis of U937 and K562 human leukemic cell lines. Exponentially growing cells were cultured without treatment or in the presence or absence of camptothecin (0.3–30 μM) or taxol (1–10 μM) for 4 to 24 h, and induction of apoptosis was determined by terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL). The effect of exposure to camptothecin to promote DNA fragmentation in U937 cells is illustrated in Fig. 1A. U937 cells are exquisitely sensitive to camptothecin-induced apoptosis, with >50% of the cells showing DNA fragmentation changes after 6 h using a concentration of 3 μM (1 μg/ml). In contrast, K562 cells were completely resistant to camptothecin-induced apoptosis under these experimental conditions (Table 1). U937 cells showed moderate sensitivity to taxol-induced apoptosis at doses between 1 and 10 μM. In contrast, K562 cells showed no

Table 1
Sensitivity of U937 cells and K562 cells to apoptosis induced by camptothecin or taxol[a]

	U937 cells[b]	K562 cells
Untreated	2.5 ± 0.6	2.7 ± 2.4
Camptothecin (3 μM)	51.6 ± 25.1[c]	3.5 ± 1.6
Taxol (1 μM)	27.8 ± 14.8[c]	2.7 ± 1.7
Negative control[d]	3.1 ± 1.6	1.3 ± 1.1
Positive control[e]	88.2 ± 9.4[c]	92.9 ± 7.1[c]

[a] U937 cells (5×10^5) or K562 cells (1×10^6) were treated with camptothecin or taxol at the indicated concentration for 6 h. Untreated cells received equivalent amounts of vehicle (NaOH for camptothecin controls and DMSO for taxol controls). At the end of the culture period, cells were washed, fixed in 2% formalin, and permeabilized using 80% EtOH. Apoptosis was examined by TUNEL.

[b] Data are expressed as % apoptotic cells (mean ± SD from two to eight experiments).

[c] Significantly different from control ($P < 0.05$).

[d] For the negative controls, TdT was omitted from the labeling step in the TUNEL assay using cells treated with camptothecin or with taxol. The results for cells treated with either compound were similar and are all included in the analysis.

[e] For the positive control, fixed and permeabilized cells that did not receive camptothecin or taxol were treated with DNase (2.5 μg) for 12 min prior to the addition of TdT.

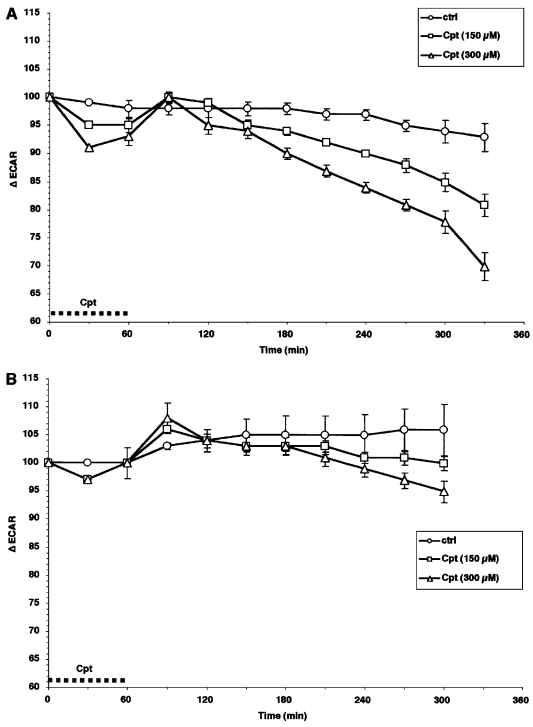

Fig. 2. Changes in extracellular acidification rate in cells treated with camptothecin. (A) U937 cells (5×10^4) or (B) K562 cells (1×10^5) remained untreated (open circles), or were exposed to camptothecin at 150 μM (open squares) or 300 μM (open triangles) using a continuous flow rate for 60 min followed by withdrawal of the compound from the running media. Control cells were treated with an equivalent amount of vehicle (NaOH). Measurements were obtained for 5 h; data points are shown at 30-min intervals. The baseline extracellular acidification rate prior to camptothecin exposure was considered equal to 100%. Data are shown as the mean ± S.E.M. for three (A) and four (B) experiments.

evidence of DNA fragmentation when treated with taxol in this dose range (Fig. 1B and Table 1).

Next, we determined if camptothecin and taxol affected the metabolic rate of these cell lines. U937 cells exposed to camptothecin (150–300 μM) with a constant flow rate for 60 min showed a dose-dependent and biphasic decrease in extracellular acidification rate (Fig. 2A). During the first 30 min, the extracellular acidification rate decreased by ~ 10%. Once the camptothecin was removed from the running media, there was a measurable recovery, with an increase in extracellular acidification rate up to 3–4% above baseline. The extracellular acidification rate gradually returned to baseline over the next 60 min, followed by a reproducible 20–30% reduction after 5 h. After 8 h, the extracellular acidification rate of U937 cells was 50% of baseline (not shown). Smaller, yet significant effects of camptothecin on extracellular acidification rate were also observable when the compound was used at 3–15 μM under the same conditions (not shown).

K562 cells treated with camptothecin were much less sensitive to camptothecin-mediated extracellular acidification rate changes (Fig. 2B). The extracellular acidification rate of untreated K562 cells remained at or near baseline levels for the 5-h experimental period. Minimal changes in extracellular acidification rate were seen in K562 cells treated with 150 μM camptothecin, although K562 cells exposed to camptothecin at 300 μM showed a decrease in extracellular acidification rate, albeit this did not fall below 80% of baseline in any experiment.

To determine if decreased metabolic rate reflected by reductions in extracellular acidification rate was a consistent, proportionate cellular response to pro-apoptotic stimuli, we examined whether taxol would induce similar alterations as those observed with camptothecin. U937 cells showed a 20% to 30% decrease in extracellular acidification rate over 5 h after a 60-min exposure to taxol (10–100 μM), but unlike the change in extracellular acidification rate seen after camptothecin treatment, that observed after treatment with taxol was not biphasic (Fig. 3A). K562 cells treated with taxol for 60 min did not show significant alterations in extracellular acidification rate during the first 5 h (Fig. 3B) or during the complete 8-h experimental period (not shown).

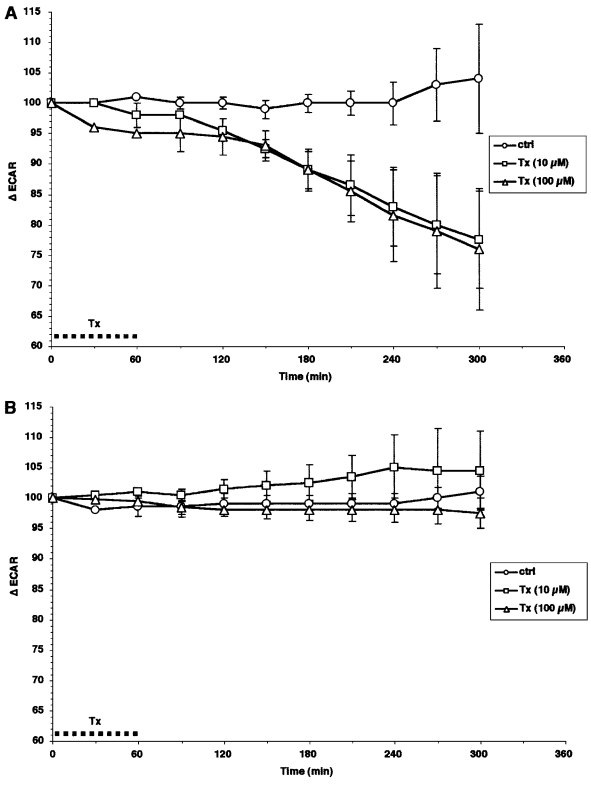

Fig. 3. Changes in extracellular acidification rate in cells treated with taxol. (A) U937 cells (5×10^4) or (B) K562 cells (1×10^5) remained untreated (open circles), or were exposed to taxol at 10 μM (open squares) or 100 μM (open triangles) using a continuous flow rate for 60 min followed by withdrawal of the compound from the running media. Control cells were treated with an equivalent amount of vehicle (DMSO). Measurements were obtained for 5 h; data points are shown at 30-min intervals. The baseline extracellular acidification rate prior to taxol exposure was considered equal to 100%. Data are shown as the means ± SD for two (A) and four (B) experiments.

To determine if the alterations in extracellular acidification rate were nonspecific changes associated with cell death, we examined the effects of the metabolic inhibitor, sodium azide, on extracellular acidification rate. As was the case with camptothecin or taxol treatment, U937 cells treated with sodium azide showed a dose-dependent change in extracellular acidification rate (Fig. 4). When sodium azide was used at relatively low concentrations (1.5 mM or 0.1 mg/ml), there was a rapid, but transient increase in extracellular acidification rate followed by a prolonged decrease. When sodium azide was used at higher concentrations (150 mM), the cells showed a rapid decrease in extracellular acidification rate to levels below 50% of baseline, followed by a more gradual decline to levels equivalent to 15–20% of baseline after 5 h. Continuous exposure to sodium azide caused an irreversible decrease in extracellular acidification rate (Fig. 4A). However, in contrast to the results seen in cells treated with camptothecin or taxol, when cells were exposed to sodium azide for less than 1 h, the effects were largely reversible and the extracellular acidification rate returned to levels near the original baseline within 30 min (Fig. 4B).

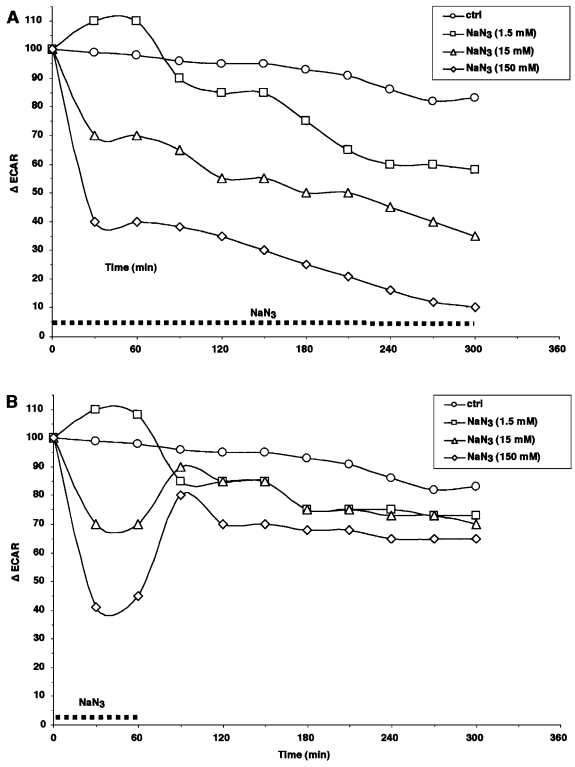

Fig. 4. Changes in extracellular acidification rate in cells treated with sodium azide. U937 cells (5×10^4) remained untreated (open circles), or were exposed to sodium azide at 1.5 mM (open squares), 15 mM (open triangles), or 150 mM (open diamonds) using a continuous flow rate for (A) 5 h or (B) 1 h, followed by withdrawal of the compound from the running media. Measurements were obtained for 5 h; data points are shown at 30-min intervals. The baseline extracellular acidification rate prior to sodium azide exposure was considered equal to 100%.

Table 2
Amiloride inhibits camptothecin-induced alkalinization of U937 cells[a]

Treatment	Extracellular acidification rate[b]
None	100
Camptothecin	86.5 + 2.9[c]
Amiloride + camptothecin	99.6 + 1.7[c]

[a] U937 cells (5×10^4) were allowed to stabilize in the Cytosensor™ and treated with 5-*N*-methyl-*N*-isobutyl amiloride (10 μM) for 30 min prior to the addition of camptothecin (300 μM) using a continuous flow rate for an additional 60 min. The compound was then withdrawn from the running media and measurements were obtained for 5 h. The baseline extracellular acidification rate prior to any treatment was considered equal to 100%.

[b] Data are expressed as the mean ± S.E.M. of replicate samples. Similar results were obtained in three independent experiments.

[c] The difference between the amiloride-treated and untreated group was statistically significant ($P < 0.05$).

Finally, to determine if the decreases in extracellular acidification rate in response to pro-apoptotic stimuli were mediated by the Na/H antiporter, cells were pre-incubated with amiloride (10 μM), an inhibitor of the Na^+/H^+ antiporter, for 30 min prior to exposure to camptothecin. Amiloride inhibited the camptothecin-induced decrease in extracellular acidification rate (Table 2), supporting a role for the Na^+/H^+ antiporter in cellular alkalinization in response to pro-apoptotic stimuli.

4. Discussion

The role of intracellular acidification or alkalization in the execution phase of apoptosis is not well understood. It has been claimed that apoptosis is modulated or perhaps even triggered by changes in intracellular pH. Several reports correlate apoptosis with intracellular acidification (Furlong et al., 1997; Meisenholder et al., 1996; Pardhasaradhi et al., 1997; Shrode et al., 1997; Thangaraju et al., 1999), while others relate this process with intracellular alkalinization (Benson et al., 1999; Dai et al., 1998).

Intracellular pH is known to change through transfer of protons from the cytoplasmic buffer pools to the extracellular environment as a pulse during a transition state (McConnell et al., 1992). Monitoring the extracellular acidification rates can be used in principle as a generic method to detect these physiological changes. To examine how pro-apoptotic stimuli affected cellular acidification or alkalinization, we used a Cytosensor™ microphysiometer to evaluate real-time changes in extracellular acidification rate as a measure of metabolic alterations of leukemic cell lines following exposure to camptothecin or taxol, compounds that promote apoptosis through distinct mechanisms (inhibition of topoisomerases and inhibition of mitotic spindle assembly, respectively). During the early phase of exposure to the apoptotic drugs, U937 cells showed a gradual decrease in extracellular acidification rate. This could be due to the conversion from glycolytic to aerobic metabolism, resulting

in reduced proton production for each ATP molecule (Owicki and Parce, 1992). Upon removal of camptothecin or taxol, a rebound of the extracellular acidification rate was observed, followed by a gradual decline, suggesting a direct effect of altered Na^+/H^+ exchange. These changes in the acid production may reflect the combined effect of glucose utilization and Na^+/H^+ antiporter activation. This was not peculiar to U937 cells alone, as Jurkat cells, which also are sensitive to camptothecin-induced apoptosis, showed a similar rebound of the extracellular acidification rate followed by a gradual decline (data not shown). When U937 cells were treated with the Na^+/H^+ antiporter inhibitor amiloride prior to exposure to camptothecin, the predicted change in extracellular acidification rate was inhibited, suggesting that the antiporter is involved in the pro-apoptotic process. Our microphysiometry results are in agreement with those of Li and Eastman (1995), who demonstrated that a functional antiporter pump is necessary for apoptosis, as well as those of Cobo et al. (1998), who identified the Na^+/H^+ exchange activity as crucial to the apoptotic process and showed that amiloride delayed the onset of apoptosis and reduced endonuclease activity and DNA fragmentation in camptothecin-treated HL-60 cells. Yet, there are non-apoptotic mechanisms that show a decrease in the extracellular acidification rate, but the change in the extracellular acidification rate in those mechanisms is limited to the time at which the cells are exposed to the drug or the metabolic inhibitor. In the non-apoptotic mechanisms, the change in the extracellular acidification rate comes back to the normal basal level once the exposure time is over. However, in the present study, apoptosis inducing drugs initiated the decrease in the extracellular acidification rate that was irreversible upon removal of the drugs. It is possible that increased cellular metabolism and extracellular acidification also contributed to induction of apoptosis by camptothecin (Gabr et al., 1997; Goossens et al., 2000). Camptothecin and its analogues can exist in two forms: carboxylate and lactone. An acidic pH (<4) favors the maintenance of the ring-closed lactone form of camptothecin, which is the most active configuration of the drug (Burke and Mi, 1993). In addition, the steady-state uptake of camptothecin in leukemia cells was shown to increase proportionally with the decrease of the pH of the incubation medium (Gabr et al., 1997). At physiological pH such as that in the running buffer, the equilibrium would favor the less toxic carboxylate form. However, in a more acidic intracellular environment, some of the camptothecin could revert to the lactone form and persist within the cell. Thus, the observation that low pH potentiates camptothecin cytotoxicity by two- to threefold (Gabr et al., 1997) may arise from the combination of three factors: (i) limited opening of the lactone form of the drug; (ii) enhancement of drug accumulation; and (iii) stimulation of endonucleases, all three events being dependent on the action of the drug on intracellular pH.

Basal extracellular acidification rates depend on the energy-producing metabolic pathways, glycolysis, and aerobic respiration. Both metabolic pathways produce protons through formation of the acidic byproducts lactic acid and CO_2, respectively. Intracellular pH (pH_i) is altered during a variety of physiological processes. The transition from one steady-state pH_i to a higher steady-state pH_i involves the transfer of protons from the cytoplasmic buffer to the extracellular environment as a pulse during the transition. It is possible that during the initial exposure to camptothecin, the extracellular acidification rate represents the sum of cellular glycolytic and respiratory activity, and is therefore a measure of cellular metabolic activity. The final outcome would be accumulation of H^+ ions, which in turn would activate the Na^+/H^+ exchange system leading to Na^+ and Ca^+ overload, and perhaps initiation of cytotoxic events.

Reductions in extracellular acidification rate below the initial basal level may be predictive of apoptosis. A recent study of tumor necrosis factor-α (TNF-α) effects on the TF-1 human erythroleukemia cell line showed extracellular acidification rate correlated well with DNA fragmentation events associated with apoptosis (Baxter et al., 1999). Similarly, our results show that the change in extracellular acidification rate of U937 and K562 cells treated with camptothecin or taxol correlated with the capacity of these compounds to promote DNA fragmentation.

Early events in apoptosis may depend on the cell type as well as the pro-apoptotic compound. U937 cells and K562 cells, which are differentially sensitive to camptothecin and taxol, showed a decrease in extracellular acidification rate that was consistent with their respective sensitivities to the compounds over 8-h experimental periods. It is noteworthy that in the susceptible U937 cells, apoptosis was detectable within 30 min of camptothecin or taxol treatment when measured by loss of membrane asymmetry (binding of Annexin V), whereas DNA fragmentation was detectable only after 2–3 h when measured by TUNEL. Similar results were reported in U937 cells by Chan et al. (1998) and in Jurkat cells by Span et al. (2002). More recently, it was shown that DNA strand breaks were detectable in CHO cells within 1 h of treatment using the alkaline comet assay (Godard et al., 2002). Finally, using a novel method called stathmo-apoptosis, the rate of apoptosis in HL-60 cells treated with camptothecin was shown to be approximately 8%/h for the first 8 h (Smolewski et al., 2002). Together, these data indicate that treatment of susceptible cells with camptothecin or taxol for 1 h is sufficient to initiate the events leading to apoptosis.

In K562 cells, the expression of $p210^{bcr\text{-}abl}$ cells may contribute to their resistance to drug-induced apoptosis (Bedi et al., 1995). Furthermore, a delayed response in cytotoxicity following pulsed or continuous exposure of K562 cells to taxol has been observed by Gangemi et al. (2000). In their study, taxol-mediated apoptosis was evident only after 48 h and was prominent after 72 h of treatment. These investigators determined that the molecular basis of the delayed response of K562 cells to taxol was downstream from phosphorylation of bcl-2 and upstream of

caspase-3 activation. In our microphysiometry experiments, cells were exposed to taxol for only 30–60 min, metabolic changes were measured for 5–8 h, and DNA fragmentation was measured after 6 to 24 h. Thus, the fact that taxol did not cause any measurable changes in extracellular acidification rate or DNA fragmentation in K562 cells may be due to the inability to activate caspase-3 within those time frames.

The pattern of changes in extracellular acidification rate that follows exposure to pro-apoptotic compounds appears to be unique. When cells were exposed to sodium azide, the effects on extracellular acidification rate were distinctly different. Sodium azide can protect cells from lethal free radical oxidation when used at very low concentrations (1.5 mM) (Hansson et al., 1996). However, at higher concentrations (150 mM), it is cytotoxic and can promote necrosis by inhibition of energy metabolism (Figiel and Kaczmarek, 1997; Franek et al., 1992; Kumi-Diaka et al., 1999; Nakagawa et al., 1996; Ohyagi et al., 2000). In contrast to the irreversible changes in extracellular acidification rate seen with the pro-apoptotic compounds camptothecin and taxol, a brief exposure of U937 cells to sodium azide led to a decrease in the extracellular acidification rate that was reversible when exposure was terminated. These results are in general agreement with those of Duranteau et al. (1998) who reported that removal of sodium azide was associated with progressive recovery from inhibition of cardiomyocyte contractile motion, except at the highest concentration (150 mM), where recovery was not evident.

In summary, our data suggest that the susceptibility of cells to pro-apoptotic compounds is proportional to changes in cellular metabolism. These results further validate the use of microphysiometry to monitor early events during the progression of apoptosis. Moreover, this technique may prove useful as a means to screen novel compounds for apoptotic activity.

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References

Baxter, G.T., Kuo, R.C., Jupp, O.J., Vandenabeele, P., MacEwan, D.J., 1999. Tumor necrosis factor-alpha mediates both apoptotic cell death and cell proliferation in a human hematopoietic cell line dependent on mitotic activity and receptor subtype expression. J. Biol. Chem. 274, 9539–9547.

Bedi, A., Barber, J.P., Bedi, G.C., el-Deiry, W.S., Sidransky, D., Vala, M.S., Akhtar, A.J., Hilton, J., Jones, R.J., 1995. BCR-ABL-mediated inhibition of apoptosis with delay of G2/M transition after DNA damage: a mechanism of resistance to multiple anticancer agents. Blood 86, 1148–1158.

Benson, R.S., Dive, C., Watson, A.J., 1999. Cytoplasmic acidification is not an effector mechanism of VP16 or DEX-induced apoptosis in CEM T leukaemia cells. J. Cell Sci. 112, 1755–1760.

Burke, T.G., Mi, Z., 1993. Preferential binding of the carboxylate form of camptothecin by human serum albumin. Anal. Biochem. 212, 285–287.

Chan, A., Reiter, R., Wiese, S., Fertig, G., Gold, R., 1998. Plasma membrane phospholipid asymmetry precedes DNA fragmentation in different apoptotic cell models. Histochem. Cell Biol. 110, 553–558.

Chinnaiyan, A.M., Tepper, C.G., Seldin, M.F., O'Rourke, K., Kischkel, F.C., Hellbardt, S., Krammer, P.H., Peter, M.E., Dixit, V.M., 1996. FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. J. Biol. Chem. 271, 4961–4965.

Cobo, J.M., Garcia-Canero, R., Valdez, J.G., Barrasso, A.M., Sailer, B.L., Crissman, H.A., 1998. Attenuation of apoptotic DNA fragmentation by amiloride. J. Cell Physiol. 175, 59–67.

Dai, H.Y., Tsao, N., Leung, W.C., Lei, H.Y., 1998. Increase of intracellular pH in p53-dependent apoptosis of thymocytes induced by gamma radiation. Radiat. Res. 150, 183–189.

Duranteau, J., Chandel, N.S., Kulisz, A., Shao, Z., Schumacker, P.T., 1998. Intracellular signaling by reactive oxygen species during hypoxia in cardiomyocytes. J. Biol. Chem. 273, 11619–11624.

Figiel, I., Kaczmarek, L., 1997. Orthovanadate induces cell death in rat dentate gyrus primary culture. NeuroReport 8, 2465–2470.

Franek, F., Vomastek, T., Dolnikova, J., 1992. Fragmented DNA and apoptotic bodies document the programmed way of cell death in hybridoma cultures. Cytotechnology 9, 117–123.

Furlong, I.J., Ascaso, R., Lopez Rivas, A., Collins, M.K., 1997. Intracellular acidification induces apoptosis by stimulating ICE-like protease activity. J. Cell Sci. 110, 653–661.

Gabr, A., Kuin, A., Aalders, M., El-Gawly, H., Smets, L.A., 1997. Cellular pharmacokinetics and cytotoxicity of camptothecin and topotecan at normal and acidic pH. Cancer Res. 57, 4811–4816.

Gangemi, R.M., Santamaria, B., Bargellesi, A., Cosulich, E., Fabbi, M., 2000. Late apoptotic effects of taxanes on K562 erythroleukemia cells: apoptosis is delayed upstream of caspase-3 activation. Int. J. Cancer 85, 527–533.

Godard, T., Deslandes, E., Sichel, F., Poul, J.M., Gauduchon, P., 2002. Detection of topoisomerase inhibitor-induced DNA strand breaks and apoptosis by the alkaline comet assay. Mutat. Res. 520, 47–56.

Goossens, J., Henichart, J.P., Dassonneville, L., Facompre, M., Bailly, C., 2000. Relation between intracellular acidification and camptothecin-induced apoptosis in leukemia cells. Eur. J. Pharm. Sci. 10, 125–131.

Gottlieb, R.A., Nordberg, J., Skowronski, E., Babior, B.M., 1996. Apoptosis induced in Jurkat cells by several agents is preceded by intracellular acidification. Proc. Natl. Acad. Sci. U. S. A. 93, 654–658.

Hansson, M., Asea, A., Ersson, U., Hermodsson, S., Hellstrand, K., 1996. Induction of apoptosis in NK cells by monocyte-derived reactive oxygen metabolites. J. Immunol. 156, 42–47.

Kumi-Diaka, J., Nguyen, V., Butler, A., 1999. Cytotoxic potential of the phytochemical genistein isoflavone (4′,5′,7-trihydroxyisoflavone) and certain environmental chemical compounds on testicular cells. Biol. Cell 91, 515–523.

Li, J., Eastman, A., 1995. Apoptosis in an interleukin-2-dependent cytotoxic T lymphocyte cell line is associated with intracellular acidification. Role of the Na(+)/H(+)-antiport. J. Biol. Chem. 270, 3203–3211.

Madshus, I.H., 1988. Regulation of intracellular pH in eukaryotic cells. Biochem. J. 250, 1–8.

McConnell, H.M., Owicki, J.C., Parce, J.W., Miller, D.L., Baxter, G.T., Wada, H.G., Pitchford, S., 1992. The cytosensor microphysiometer: biological applications of silicon technology. Science 257, 1906–1912.

Meisenholder, G.W., Martin, S.J., Green, D.R., Nordberg, J., Babior, B.M., Gottlieb, R.A., 1996. Events in apoptosis. Acidification is downstream of protease activation and BCL-2 protection. J. Biol. Chem. 271, 16260–16262.

Modiano, J.F., Ritt, M.G., Wojcieszyn, J., Smith III, R., 1999. Growth arrest of melanoma cells is differentially regulated by contact inhibition and serum deprivation. DNA Cell Biol. 18, 357–367.

Nakagawa, Y., Moldeus, P., Moore, G.A., 1996. Relationship between mitochondrial dysfunction and toxicity of propyl gallate in isolated rat hepatocytes. Toxicology 114, 135–145.

Ohyagi, Y., Yamada, T., Nishioka, K., Clarke, N.J., Tomlinson, A.J., Naylor, S., Nakabeppu, Y., Kira, J., Younkin, S.G., 2000. Selective increase in cellular A beta 42 is related to apoptosis but not necrosis. NeuroReport 11, 167–171.

Owicki, J.C., Parce, J.W., 1992. Biosensors based on the energy metabolism of living cells: the physical chemistry and cell biology of extracellular acidification. Biosens. Bioelectron. 7, 255–272.

Pardhasaradhi, B.V., Khar, A., Srinivas, U.K., 1997. Effect of anti-apoptotic genes and peptide inhibitors on cytoplasmic acidification during apoptosis. FEBS Lett. 411, 67–70.

Pittman, R.N., Wang, S., DiBenedetto, A.J., Mills, J.C., 1993. A system for characterizing cellular and molecular events in programmed neuronal cell death. J. Neurosci. 13, 3669–3680.

Raffray, M., Cohen, G.M., 1997. Apoptosis and necrosis in toxicology: a continuum or distinct modes of cell death? Pharmacol. Ther. 75, 153–177.

Schulze-Osthoff, K., Ferrari, D., Los, M., Wesselborg, S., Peter, M.E., 1998. Apoptosis signaling by death receptors. Eur. J. Biochem. 254, 439–459.

Shrode, L.D., Tapper, H., Grinstein, S., 1997. Role of intracellular pH in proliferation, transformation, and apoptosis. J. Bioenerg. Biomembranes 29, 393–399.

Smolewski, P., Grabarek, J., Lee, B.W., Johnson, G.L., Darzynkiewicz, Z., 2002. Kinetics of HL-60 cell entry to apoptosis during treatment with TNF-alpha or camptothecin assayed by the stathmo-apoptosis method. Cytometry 147, 143–149.

Span, L.F., Pennings, A.H., Vierwinden, G., Boezeman, J.B., Raymakers, R.A., de Witte, T., 2002. The dynamic process of apoptosis analyzed by flow cytometry using Annexin-V/propidium iodide and a modified in situ end labeling technique. Cytometry 47, 24–31.

Thangaraju, M., Sharma, K., Leber, B., Andrews, D.W., Shen, S.H., Srikant, C.B., 1999. Regulation of acidification and apoptosis by SHP-1 and Bcl-2. J. Biol. Chem. 274, 29549–29557.