Distinct Modes of Cell Death Induced by Different Reactive Oxygen Species

AMINO ACYL CHLORAMINES MEDIATE HYPOCHLOROUS ACID-INDUCED APOPTOSIS*

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Oxidants derived from inflammatory phagocytes compose a key element of the host immune defense system and can kill mammalian cells by one of several different mechanisms. In this report, we compare mechanisms of cell death induced in human B lymphoma cells by the inflammatory oxidants superoxide, H$_2$O$_2$, and HOCl. The results indicate that the mode of cell death induced depends on the nature of the oxidant involved and the medium in which the cells are treated. When human Burkitt’s lymphoma cells are exposed to superoxide anion, generated as a flux from xanthine and xanthine oxidase, the cells die by a non-apoptotic mechanism (pyknosis/necrosis) identical to that seen when cells are treated with a bolus of reagent H$_2$O$_2$. Addition of superoxide dismutase has no effect, whereas catalase is completely protective, indicating that exogenously generated superoxide kills cells entirely through its dismutation into H$_2$O$_2$. In contrast, cells treated in culture media with reagent HOCl die largely by apoptosis. HOCl-induced apoptosis is mediated by aminoacyl chloramines generated in the culture media and can be mimicked by treatment of cells with taurine chloramine or with long lived chloramines generated from modified Lys or Arg. The results suggest that in a physiological milieu in which O$_2^-$ and H$_2$O$_2$ are the main oxidants being formed, the principal form of cell death may be necrotic, and under inflammatory conditions in which HOCl is generated, apoptotic cell death may predominate.

Oxidants (reactive oxygen species, ROS) are generated in high levels by activated phagocytes (neutrophils, monocytes, and macrophages) in inflammatory tissues (1, 2). These oxidants compose an important element of the host defense against bacteria and tumor cells (3). However, secreted ROS can have detrimental side effects as well, causing tissue damage and contributing to the development or progression of numerous different diseases (4). The primary oxidants generated by all normal phagocytes are the superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), which can, in the presence of reducing metals, go on to form hydroxyl and other free radicals. In addition, monocytes and neutrophils contain high levels of the enzyme myeloperoxidase which catalyzes formation of the potent oxidant, hypochlorous acid (HOCl). HOCl can then go on to react with extracellular amino acids to generate chloramines, which maintain some of the oxidizing potential of HOCl but are not as potent as or as broadly reactive (5–7). Each of these different ROS have molecular characteristics that account for their different levels of reactivity with cellular and extracellular macromolecules.

The studies presented here focus on mechanisms of cell death induced by inflammatory oxidants. Mammalian cells can die by one of several different defined pathways, the most common of which are apoptosis and necrosis. Apoptosis is characterized by a discrete set of biochemical steps and morphological changes including activation of caspases, translocation of phosphatidylserine from the inner to the outer layer of the plasma membrane, chromatin condensation, and fragmentation into apoptotic bodies (8, 9). In contrast, cells that die by necrosis swell and then lyse, releasing their contents into the extracellular space (10). It is thought that death by apoptosis is physiologically advantageous because early apoptotic cells are cleared by phagocytosis and subsequent intracellular degradation (11). In this manner, apoptotic cells are removed without causing damage to the surrounding tissue. In contrast, necrotic cells are thought to promote an inflammatory response caused by the leakage of intracellular proteins and nucleic acids prior to phagocytosis. In support of this theory, we found that B lymphoma cells treated with H$_2$O$_2$ die by a non-apoptotic mechanism and are not phagocytosed by macrophages until after they begin to lose their plasma membrane integrity (12). In contrast, early apoptotic cells induced by chemotherapy drugs underwent phagocytosis while their membranes were still intact.

Previous research (13–21) has demonstrated that exogenously added H$_2$O$_2$ can induce either apoptosis or necrosis, depending on the concentration of H$_2$O$_2$, the cell type being studied, and the level of ATP in the cells. Relatively little has been reported on mechanisms of induction of cell death from exogenously generated O$_2^-$ or HOCl (22), such as would be produced by activated neutrophils, and we are unaware of any study that does a direct comparison of the modes of cell death induced by the different oxidants in the same cell type. This report compares mechanisms of B lymphoma cell death induced by exogenous O$_2^-$, H$_2$O$_2$, and HOCl. The data reveal that O$_2^-$ kills cells solely through its dismutation into H$_2$O$_2$, and...
both of these ROS kill by a non-apoptotic mechanism referred to as pyknosis/necrosis (20). In stark contrast, HOCl induces either necrosis or apoptosis depending on the cell environment; in buffered saline, cell death is entirely by rapid necrosis, and in growth media, cell death is primarily apoptotic. We demonstrate further that long lived aminoacyl chloramines mediate HOCl-induced apoptosis.

MATERIALS AND METHODS

Cells and Treatments—The Burkitt’s lymphoma cell lines JLP-119 and BL-41 were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 0.5 mg/ml streptomycin, 0.5 mg/ml gentamicin, and 250 μg/ml amphotericin B. The JLP-119 and BL-41 cell lines were previously cloned into a lentiviral vector expressing a luciferase reporter gene (26) and were grown at 37°C in 5% CO₂ as described previously (20). The cells were incubated at 37°C for the times indicated in the figure legends. A flux of O₂ and H₂O₂ was generated by adding xanthine (X, 50–400 μM) to the cells in culture media 1 h prior to addition of xanthine oxidase (XO, 20 μl/million cells; Sigma catalog number X-4500), and cells were incubated for the times indicated in the figure legends. Reagent NaOCl (1–500 μM; Aldrich) or H₂O₂ was added to the cell suspensions, and the cells were incubated for the times indicated in the figure legends. Concentrations of stock solutions were based on molar extinction coefficients of 0.05 cm⁻²/mmol NaOCl at 292 nm (23) and of 50 m M cm⁻¹ for H₂O₂ at 240 nm (24). Chloramphenicol toxicity studies were carried out by mixing each amino acid (2 mM in PBS on ice) with NaOCl (50–500 μM) and then adding the mixtures (1 ml) immediately (<30 s) to cell pellets to get a final density of 5 × 10⁵ cells/ml. After a 1-h incubation at 37°C, 1 ml of complete media was added to each well, and cells were incubated for an additional 7 h.

Assessment of Apoptosis Using FACSscan Analysis—To determine the percentage of cells expressing phosphatidylserine (PS) on the exofacial surface of the plasma membrane, cells were centrifuged and resuspended in fresh media to achieve a culture density of 5 × 10⁵ cells/ml. Reagent H₂O₂ (50–200 μM) was added to the cell suspensions, and the cells were incubated at 37°C for the times indicated in the figure legends. A flux of O₂ and H₂O₂ was generated by adding xanthine (X, 50–400 μM) to the cells in culture media 1 h prior to addition of xanthine oxidase (XO, 20 μl/million cells; Sigma catalog number X-4500), and cells were incubated for the times indicated in the figure legends. Reagent NaOCl (1–500 μM; Aldrich) or H₂O₂ was added to the cell suspensions, and the cells were incubated for the times indicated in the figure legends. Concentrations of stock solutions were based on molar extinction coefficients of 0.05 cm⁻²/mmol NaOCl at 292 nm (23) and of 50 m M cm⁻¹ for H₂O₂ at 240 nm (24). Chloramphenicol toxicity studies were carried out by mixing each amino acid (2 mM in PBS on ice) with NaOCl (50–500 μM) and then adding the mixtures (1 ml) immediately (<30 s) to cell pellets to get a final density of 5 × 10⁵ cells/ml. After a 1-h incubation at 37°C, 1 ml of complete media was added to each well, and cells were incubated for an additional 7 h.

Morphological Assessment of Cell Death Using Hoechst/Propidium (PI) Nuclear Staining and Fluorescence Microscopy—Cells (5 × 10⁶ cells/ml) were incubated for 15 min at 37°C with Hoechst 33342 dye (5 μg/ml in PBS), centrifuged, washed once in PBS, and then resuspended at ~2.5 × 10⁵ cells/ml. Propidium iodide (PI, 50 μg/ml from a 1 mg/ml stock in PBS) was added just before microscopy. Cells were visualized using fluorescence microscopy as described previously (20). A minimum of 200 cells was counted, and cell morphology was classified as follows: (i) live cells (normal nuclei, blue chromatin with organized structure); (ii) membrane-intact apoptotic cells (bright blue chromatin which is highly condensed, margined, or fragmented); (iii) membrane-permeable apoptotic cells (bright red chromatin, highly condensed or fragmented); (iv) necrotic cells (red, enlarged nuclei with smooth normal structure); and (v) pyknotic/necrotic cells (dense, red, slightly condensed nuclei with no fragmentation).

RESULTS

In previous studies, we found that the mode of B lymphoma cell death induced by H₂O₂ is non-apoptotic (20). We refer to the cell death induced by H₂O₂ in these cells as pyknosis/necrotic because the nuclei are slightly condensed, whereas there are no signs of classical apoptotic changes. Quantification of H₂O₂-induced cell death (18 h of incubation) in two different Burkitt’s lymphoma (BL) cell lines is shown in Fig. 1 and demonstrates again that only a low level of apoptosis is induced by cytotoxic concentrations of H₂O₂, which for these cell lines is from 50 to 200 μM, with the BL-41 cell line showing greater resistance to H₂O₂-induced cell death (Fig. 1B) compared with JLP-119 cells (Fig. 1A). Because most H₂O₂ in inflammatory conditions is derived from XO, we set out to determine whether exogenously generated O₂− causes a similar or different mode of cell death. This question is of interest because O₂− has the potential to promote hydroxyl radical formation through the Haber-Weiss reaction, in addition to forming H₂O₂. The results in Fig. 2 show that cell death induced by exposure to O₂− generated from XO andXA was primarily pyknotic/necrotic at all treatment levels, similar to H₂O₂. Addition of superoxide dismutase to the incubation medium had no effect on the type or level of cell death induced, whereas catalase inhibited completely cell death induced by XAOX. Similar results were obtained with JLP-119 cells (data not shown). The results show...
that cell death from exogenously generated $O_2$ derives mostly from formation of $H_2O_2$.

One of the main oxidants generated during an inflammatory response involving neutrophils and/or undifferentiated monocytes is $HOCl$, formed from the action of myeloperoxidase on $H_2O_2$ and $Cl^-$ (2). When we exposed BL cells to reagent $HOCl$, we found that, in stark contrast to $H_2O_2$ and $O_2$, a high level of apoptosis was induced (Fig. 3). The other main form of cell death induced from $HOCl$ was classical necrosis, with only a low level of pyknosis/necrosis seen (determined from the nuclear morphology). Note that BL-41 cells, which are somewhat more resistant to oxidant-induced killing than JLP-119 cells, incurred a higher level of necrosis compared with JLP-119 cells, which were mostly apoptotic. Apoptosis was confirmed by measuring activation of caspase-3, as determined from cleavage of the caspase-3 pro-enzyme by Western blot immunoassay (Fig. 4) and by measuring annexin V binding to externalized PS (see below).

To determine why $H_2O_2$ and $HOCl$ induce such different forms of cell death, we compared the effects of these two oxidants on GSH and ATP levels in the cells. As shown in Fig. 5, the differences cannot be attributed to different effects on cellular GSH levels. Concentrations of $H_2O_2$ and $HOCl$ that induce roughly 60–80% cell death in the cells caused mild to moderate reductions in cellular GSH levels ($\leq 20\%$ decrease in JLP-119 cells and $\leq 35\%$ decrease in BL-41 cells), and the effects of $H_2O_2$ and $HOCl$ on cellular ATP levels were markedly different (Fig. 6). As we saw previously (20), $H_2O_2$ caused a complete and irreversible loss of intracellular ATP within 15 min after addition to the cells. However, $HOCl$ caused only a mild and transient drop in ATP levels that was restored to control levels within 1 (BL-41 cells) to 2 h (JLP-119 cells). The ability of $HOCl$ to induce apoptosis correlates with its ability to induce significant cell damage without depleting the cells of ATP and is consistent with reports that ATP must be main-
tained at or above 25% of control levels in order for apoptosis to occur (19–21, 29).

Previous studies into the mechanism of cytotoxicity induced by HOCl found that the main mode of cell death is necrotic (30), with endothelial cells undergoing only low levels of apoptosis in response to HOCl treatment (22). We noted that those earlier studies were carried out by treating the cells in buffer, not growth media, so we set out to determine whether the medium in which the cells are treated influences the mechanism of cell killing. Note that these and all subsequent experiments were performed with BL-41 cells, and apoptosis was quantified primarily by measuring binding of annexin V followed by FACS analysis to detect cells that have externalized plasma membrane phosphatidylserine, a hallmark of apoptosis. BL-41 cells were selected because they express significantly higher levels of exofacial PS than JLP-119 cells. Control studies showed that the annexin V assay gave nearly identical results for the quantification of apoptotic and necrotic cells as the assay for nuclear morphology (see "Materials and Methods" for experimental details). The results in Fig. 7 show that when BL-41 cells are treated with HOCl in PBS/glucose, cell death is entirely by necrosis. In contrast, when the cells are treated either in com-
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Fig. 6. ATP levels in Burkitt’s lymphoma cells after exposure to H$_2$O$_2$ or HOCl. JLP-119 cells (A) and BL-41 cells (B) were exposed to H$_2$O$_2$ (100 μM for JLP-119 or 200 μM for BL-41 cells) or HOCl (500 μM for both cell lines) for up to 2 h, without washing or exchanging the media. Cellular ATP levels were measured at the indicated times and are presented as the percentage of the ATP levels in control (untreated) cells. Control ATP levels in JLP-119 and BL-41 cells were 0.9 and 1.3 nmol/10$^6$ cells, respectively.

It is widely thought that oxidants and other cytotoxic agents induce apoptosis at low concentrations and necrosis at high concentrations (13). To determine whether HOCl might induce apoptosis in PBS if lower concentrations were employed, we treated BL-41 cells with levels of HOCl ranging from 1 to 500 μM, and we measured cell death after 8 h. The cells are relatively unstable in PBS/glucose in the absence of protein and die spontaneously when incubated for longer times. Hence, incubations in PBS were limited to 2 h after which complete media were added, and the cells were incubated for an additional 6 h. As shown in Fig. 8, we did not detect any apoptosis when cells were treated in this manner with low concentrations of HOCl in PBS/glucose.

The results in Fig. 7 demonstrate a profound effect of the media on the mode and level of cell killing by HOCl. RPMI contains several types of components including vitamins, salts, glucose, and high levels of free amino acids (6.8 mM total, which is comparable with the level of amino acids found in human plasma) (31). In initial experiments, we found that of these components, only the amino acids influenced HOCl-induced cell killing; treatment of cells in RPMI vitamins or salts gave results similar to treatment in PBS/glucose. It is well known that HOCl can react with amino acids to form chloramines (5–7) and that these can be cytotoxic (32). The mode of cell killing by aminoacyl chloramines has not been described previously. The following experiments demonstrate that induction of apoptosis by treatment of BL cells with HOCl in RPMI is mediated by formation of long lived aminoacyl chloramines. Unless otherwise noted, the experiments employed 500 μM HOCl, which causes 40–60% apoptotic cell death when cells are treated in RPMI.

First, spectrophotometric studies showed that when different amino acids (2 mM) were incubated with HOCl (500 μM) on ice, they were converted immediately (within seconds) to chloramines, which have a characteristic absorbance peak at 252 nm as described by Test et al. (6) (data not shown). At the same time, the absorbance peak for reagent HOCl at 292 nm was lost immediately. At this ratio of amino acids to HOCl (4:1), there was no detectable HOCl remaining after it was added to the amino acid mixtures.

Chloramines have different levels of stability depending on the chemistry of the modified amino group. The data in Fig. 9A show that most of the aminoacyl chloramines tested were short lived and decomposed in the course of a 30-min incubation at room temperature. These short lived chloramines are known to be derived from reaction of the α-amino group with HOCl (5, 33, 34). In contrast, the chloramine formed from the β-amino acid taurine was stable, as expected (23), and did not decompose into an aldehyde even after an overnight incubation (data not shown). The results with Lys and Arg were mixed because they have additional amino groups that can react with HOCl to form chloramines of differing stability. As shown in Fig. 9A, when the α-amino group of Lys was blocked by N-acetylation, a stable chloramine was formed on the ε-amino group. However, when the ε-amino group was blocked, an unstable α-chloramine was formed that decomposed into an aldehyde (Fig. 9B).

The finding that non-acetylated Lys decayed by more than 50% is consistent with the finding of Hazen et al. (33) that the α-amino group is more susceptible to chlorination than the ε-amino group. A similar result was obtained with Arg; when the α-amino group was blocked with an acetyl moiety, a stable chloramine was formed, whereas when the side chain guanidinium group was blocked, a relatively labile chloramine was formed. Note also that the rates of decay of the different α-chloramines are not uniform; Glu and Gln decayed extremely rapidly, whereas Ile and His decayed more slowly. Formation of aldehydes from each of the amino acids that showed chloramine decay, as shown for N-ε-acetyl-Lys in Fig. 9B and as characterized previously (33, 34), was confirmed as described under “Materials and Methods” (data not shown).

The results in Fig. 10 show the different modes of cell death induced by treating BL-41 cells with the different aminoacyl chloramines. For these experiments, each amino acid (2 mM in PBS) was mixed on ice with HOCl (100–500 μM) and then added to cells in PBS/glucose. PBS/glucose alone was used as the control. The cells were incubated for 1 h at 37 °C followed by addition of an equal volume of complete media and incubation for an additional 7 h. The mode of cell death was assessed by measuring PS externalization and permeability to propidium iodide. The control treatment with PBS/glucose for 1 h followed by incubation with media resulted in roughly 18% cell death, mostly by necrosis. This level of cell death was subtracted from the treatment groups in order to depict only the increase in cell death induced by the various HOCl-modified...
amino acids. The results demonstrate that treatment of the cells with three long lived chloramines (from taurine, N-acetyl-Lys and N-acetyl-Arg) caused cell death primarily through apoptosis. HOCl-modified Lys also caused apoptosis but only when tested at lower concentrations (100 μM); at higher concentrations (up to 500 μM), the aldehydes and subsequent breakdown products from this amino acid caused increasing amounts of necrosis. Ile and His, which formed chloramines with an intermediate half-life, induced cell death that was roughly half-apoptotic and half-necrotic. The products of HOCl plus Gln, Glu, and N-acetyl-Lys were relatively nontoxic. Control experiments showed no toxicity from free amino acids (2 mM in PBS/glucose) that had not been reacted with HOCl. These included His, Leu, Ile, Lys, Glu, Gln, Arg, taurine, N-acetyl-Lys, and complete mixtures of RPMI amino acids (Sigma) tested 1 time (total amino acid concentration of 6.8 mM).

DISCUSSION

In this report, we demonstrate two significant new findings as follows: 1) different ROS induce different modes of cell death in human B lymphoma cells, and 2) HOCl-induced apoptosis is mediated by the interaction of HOCl with amino acids in the medium to form aminoacyl chloramines. Exogenously generated \( \cdot O_2^- \) kills cells entirely through its dismutation into \( \cdot H_2O_2 \), and both \( \cdot O_2^- \) and \( \cdot H_2O_2 \) kill lymphoma cells by a non-apoptotic mechanism referred to as pyknosis/necrosis. As described previously for \( \cdot H_2O_2 \), these cells have mildly condensed (pyknotic) nuclei but show none of the classical features of apoptosis such as externalization of PS, caspase activation, formation of apo-
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Fig. 8. Concentration-response study of cell death induced by HOCl in PBS. BL-41 cells were exposed to the indicated concentrations of HOCl in PBS. At 2 h complete medium was added, and cells were incubated for an additional 6 h. Cell death was assessed by flow cytometry and reported as either necrotic or apoptotic as described in the legend to Fig. 7.

Fig. 9. Generation of chloramines and aldehydes from HOCl treatment of amino acids. A, amino acids (2 mM) were mixed with HOCl (500 μM) in PBS on ice. Chloramine formation and decay was assessed from the absorbance at 252 nm during incubation at room temperature. The results are presented as the percentage of absorbance at 252 nm at time 0. B, aldehyde formation was measured as described under "Materials and Methods" from the absorbance at 540 nm. Chloramine decay and aldehyde formation for HOCl treated N-ε-acetyl-Lys are shown.

Induction of Different Modes of Cell Death by Different ROS—The term "oxidative stress" is a catch-all term intended to describe the condition that exists when the levels of ROS produced exceed the capacity of anti-oxidant systems to remove those ROS such that excess oxidants exist in a cell or tissue. The term is non-descriptive regarding which ROS are involved. Our results indicate that the mode of cell death induced by "oxidants" depends on the nature and environment of the ROS that are involved, and this will depend on the source of the oxidative stress. Note that because our research focuses on how oxidants that are generated during inflammation induce cell death in target tumor cells, the experiments described here examine the effects of oxidants generated outside of a cell. The results cannot be employed to deduce how these same oxidants might act if generated inside of a cell, where different intracellular compartments would be affected. Under biological conditions in which O₂ and H₂O₂ are the main oxidants being formed, cell death in human B lymphoma cells is expected to be non-apoptotic, whereas if HOCl is the predominant oxidant, cell death may instead be by apoptosis. It should be pointed out that, experimentally, H₂O₂ can induce apoptosis, but this only occurs in cells where the ATP levels are maintained above a certain threshold level of roughly 25% of control ATP levels (19–21, 29). This may naturally be the case for certain cell types such as T cells, which have been shown to undergo apoptosis in response to H₂O₂ but can also be induced experimentally in B lymphoma cells by inhibiting the activation of poly(ADP-ribose) polymerase that follows H₂O₂-induced strand breakage (20, 21, 35). In fact, even high levels of H₂O₂ can induce apoptosis as long as cellular ATP levels are maintained. Incidentally, our studies also show that the same mode of cell death is induced by H₂O₂ regardless of whether the cells are exposed to a bolus (reagent) or flux (from XA/XO) of H₂O₂. Physiological conditions in which O₂⁻ and H₂O₂ predominate would include conditions of ischemia-reperfusion such as occur during a heart attack or ischemic stroke, following exposure to ionizing radiation or redox active drugs and chemicals, and during inflammatory conditions involving primarily macrophages. Due to the high levels of myeloperoxidase in human neutrophils, a significant portion of the H₂O₂ that is generated by these cells is converted to HOCl (1, 23, 36, 37). Hence, physiological conditions that lead to extensive HOCl formation will be acute and chronic inflammatory conditions involving neutrophils and monocytes (which also express myeloperoxidase).

Mechanism of HOCl-induced Cytotoxicity—Most studies of HOCl-induced cytotoxicity have been carried out on cells incubated in buffer solutions instead of cell culture media (30, 38, 39). In these studies, the mode of cell death was not specifically determined, but cytotoxicity was assayed by methods that are generally reflective of necrosis (e.g. trypan blue exclusion or Cr⁴⁺ release). In more recent studies, Vissers et al. (22) treated endothelial cells with HOCl for 15 min in buffered saline and then transferred the cells to complete media. Under these conditions, they found that at most 20% of the cells died by apoptosis and that the predominant form of cell death induced by higher levels of HOCl was by necrosis, consistent with our results. But in vivo, cells will not be exposed to HOCl in the...
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The levels of cell death shown reflect the increases over control values. The data presented represent averages from 2-3 separate experiments carried out in duplicate.

absence of a large number of molecules that will react with the HOCl, primary among which are proteins and free amino acids. Hence, we focused on the mechanisms of cell death induced by HOCl in the presence of biological media.

The novel finding that we present here is that when cells are exposed to HOCl in growth media, death is directed toward apoptosis instead of necrosis, and this induction of apoptosis is due to the formation of aminoacyl chloramines in the medium. HOCl-generated chloramines have long been known to be cytotoxic to bacteria and mammalian cells (32, 38-44), but here, too, the mechanism of cell death either was not studied or measured only loss of membrane integrity (release of Cr51). Our data show that some aminoacyl chloramines, such as from Glu and Leu, decay into aldehydes (3-carboxy-propanal and 3-methyl-1-butanal, respectively) that are relatively nontoxic to cells and protect them from HOCl toxicity, allowing neither necrosis nor apoptosis. Others decay into highly cytotoxic aldehydes that induce necrosis, as is seen from the degradation of Ala monochloramine into acetaldehyde (data not shown). Amino acids that form chloramines of intermediate half-life kill by both apoptosis and necrosis, with the necrosis probably being induced primarily by the aldehydes and/or tertiary products formed. Because all of these amino acids are present in RPMI at varying concentrations, they compete with each other for the limiting amounts of HOCl used in these studies. The net effect is that treatment of cells in either RPMI or complete media results in at least a substantial proportion of the cells dying by apoptosis instead of necrosis. Serum proteins also react with HOCl and in these studies appear to act as true scavengers, protecting the cells from HOCl toxicity such that higher levels of HOCl are required to induce cell death.

Future studies will examine the molecular targets and pathways that account for chloramine-induced apoptosis in human lymphoma cells. Rapid loss of membrane integrity (lysis) in cells treated with HOCl in PBS (as evidenced by uptake of PI) and release of Cr51 (30) indicates that HOCl toxicity derives in part from a direct attack of the oxidant on the cell membrane. HOCl is known to modify membrane lipids (52-54) as well as protein and DNA. On the other hand, aminoacyl chloramines are not as reactive as HOCl and hence are expected to have a more selective spectrum of molecular targets. The absence of lysis of cells treated with the long lived aminoacyl chloramines from taurine, Nα-acetyl-Lys, and Nα-acetyl-Arg, even at concentrations of 500 μM, suggests that the chloramines may not act on membrane lipids. Instead, proteins compose a likely target of chloramine oxidation, with cysteine thiols and the methionine sulfoether being most susceptible (55, 56). Taurine chloramine in particular has been shown to modify and inhibit activities of various proteins (38, 56-58). The critical task for apoptosis research will be to identify the specific molecular targets that initiate caspase activation and the remainder of the apoptotic cascade.

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FIG. 10. Concentration-response study of cell death induced by HOCl and chloramine (NCI) treatment. BL-41 cells were exposed to either HOCl (500 μM) or aminoacyl chloramines. After a 1-h incubation, an equal volume of complete media was added, and the cells were incubated for an additional 7 h at 37 °C. Cell death was assessed by flow cytometry following staining with FITC-annexin V and PI and reported as either necrotic or apoptotic as described under "Materials and Methods." The levels of cell death shown reflect the increases over control values. The data presented represent averages from 2-3 separate experiments carried out in duplicate.

As pointed out by Weiss and colleagues (6, 45), long lived chloramines differ significantly from HOCl because they maintain an oxidizing potential yet are stable enough to diffuse some distance before oxidizing susceptible target molecules. In vivo, the most abundant amino acid in cells and tissues is taurine (46, 47), which forms a long lived chloramine that we now show causes apoptosis. Taurine is especially abundant in neutrophils, being present at ~20 mM (40, 48), and our data would predict that the main form of neutrophil cell death should be apoptotic because much of the HOCl is scavenged by taurine to form taurine chloramine (23, 45). This prediction has been borne out by studies of Wagner et al. (49) who showed that the cell death induced in myelomonocytic HL-60 cells by H2O2 is mediated by myeloperoxidase, and the mode of cell death is apoptotic. In addition, neutrophils are known to secrete taurine into the extracellular medium (40), thereby increasing the likelihood that neutrophil-induced death in neighboring cells (e.g., target tumor cells) will be apoptotic.

We find that the short lived aminoacyl chloramines, which decay rapidly into aldehydes and tertiary products (33, 34, 50, 51), can have variable effects on cell viability. The chemical structures of 13 of the aldehyde products have been characterized by mass spectrometry by Heinecke and colleagues (33, 34, 51). Our data show that some aminoacyl chloramines, such as from Glu and Leu, decay into aldehydes (3-carboxy-propanal and 3-methyl-1-butanal, respectively) that are relatively nontoxic to cells and protect them from HOCl toxicity, allowing neither necrosis nor apoptosis. Others decay into highly cytotoxic aldehydes that induce necrosis, as is seen from the degradation of Ala monochloramine into acetaldehyde (data not shown). Amino acids that form chloramines of intermediate half-life kill by both apoptosis and necrosis, with the necrosis probably being induced primarily by the aldehydes and/or tertiary products formed. Because all of these amino acids are present in RPMI at varying concentrations, they compete with each other for the limiting amounts of HOCl used in these studies. The net effect is that treatment of cells in either RPMI or complete media results in at least a substantial proportion of the cells dying by apoptosis instead of necrosis. Serum proteins also react with HOCl and in these studies appear to act as true scavengers, protecting the cells from HOCl toxicity such that higher levels of HOCl are required to induce cell death.

Future studies will examine the molecular targets and pathways that account for chloramine-induced apoptosis in human lymphoma cells. Rapid loss of membrane integrity (lysis) in cells treated with HOCl in PBS (as evidenced by uptake of PI) and release of Cr51 (30) indicates that HOCl toxicity derives in part from a direct attack of the oxidant on the cell membrane. HOCl is known to modify membrane lipids (52-54) as well as protein and DNA. On the other hand, aminoacyl chloramines are not as reactive as HOCl and hence are expected to have a more selective spectrum of molecular targets. The absence of lysis of cells treated with the long lived aminoacyl chloramines from taurine, Nα-acetyl-Lys, and Nα-acetyl-Arg, even at concentrations of 500 μM, suggests that the chloramines may not act on membrane lipids. Instead, proteins compose a likely target of chloramine oxidation, with cysteine thiols and the methionine sulfoether being most susceptible (55, 56). Taurine chloramine in particular has been shown to modify and inhibit activities of various proteins (38, 56-58). The critical task for apoptosis research will be to identify the specific molecular targets that initiate caspase activation and the remainder of the apoptotic cascade.

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