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Initiation of Rapid, P53-Dependent Growth Arrest in Cultured Human Skin Fibroblasts by Reactive Chlorine Species

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Oxidants produced by neutrophils have been implicated in causing cancers associated with chronic inflammation. Hypochlorous acid is the most potent oxidant produced by these cells in appreciable amounts. It reacts with amines to form chloramines, which are weaker oxidants but are mutagenic. Recently, we showed that sublethal doses of hypochlorous acid increased levels of the transcription factor protein 53 (p53) and the wild-type activating fragment-1/cyclin-dependent kinase inhibitory protein-1 (WAF1/CIP1) in cultured human skin fibroblasts. WAF1/CIP1 is an important intermediate in the pathway leading to growth arrest. We now show that low doses of hypochlorous acid and physiological chloramines lead to an inhibition of both DNA synthesis and division of cultured human skin fibroblasts. Inhibition of DNA synthesis occurred within 1 h of hypochlorous acid treatment, was maintained for 24 h, and returned to a normal rate after 48 h. Cell division was inhibited by hypochlorous acid and chloramines for 48 h and returned to normal 72 h after treatment. Growth arrest was dependent on p53 because it was blocked when cells were transfected with a p53-binding oligonucleotide. We propose that reactive chlorine species will initiate WAF1/CIP1-dependent growth arrest that will counteract their mutagenic effects and minimize the possibility of the malignant transformation of cells surrounding sites of inflammation.

Key Words: **p53; growth arrest; hypochlorous acid; chloramines; neutrophils; myeloperoxidase.**

Chronic inflammation is associated with an increased incidence of malignancy (1, 2). This association has led to the proposal that inflammatory leukocytes contribute to carcinogenesis. Indeed, stimulated neutrophils *in vitro* induce DNA damage in neighboring cells (3) and have been found to promote malignant tumors in mice (4). When neutrophils accumulate at sites of inflammation, they generate an array of reactive oxidants, including superoxide and hydrogen peroxide (5). Coreleased myeloperoxidase uses hydrogen peroxide to oxidize chloride to hypochlorous acid (6). This strong oxidant is bactericidal and has been proposed to be responsible for the tissue damage that occurs at sites of inflammation (7, 8). Hypochlorous acid reacts with a wide range of biomolecules, particularly thiols, methionine, and amines (9). Chloramines are formed from the reaction of hypochlorous acid with amines (10, 11). These compounds retain some of the oxidizing properties of hypochlorous acid and can be cytotoxic (10, 12, 13). The oxidants neutrophils produce may be responsible for promoting various cancers because they damage DNA (3, 14) and are mutagenic (13, 15). Although hypochlorous acid has been reported not to cause DNA strand breaks (14) or to be mutagenic (13), exposure of calf thymus DNA to hypochlorous acid leads to extensive DNA base modification, including formation of chlorinated bases (16). In contrast to hypochlorous acid, chloramines are mutagenic (13).

Recently, we showed that hypochlorous acid generated by neutrophils initiated a signal transduction pathway in cultured human skin fibroblasts that activated the transcription factor protein 53 (p53)[2] (17).

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[2] Abbreviations used: p53, transcription factor protein 53; WAF1/CIP1, wild-type activating fragment-1/cyclin-dependent kinase inhibitory protein-1.

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This led to synthesis of wild-type activating fragment-1/cyclin-dependent kinase inhibitory protein-1 (WAF1/CIP1), also known as p21. p53 is a tumor suppressor protein that becomes elevated in cells after exposure to DNA-damaging agents (18). It migrates from the cytoplasm to the nucleus where it transcriptionally activates genes, such as WAF1/CIP1, that promote growth arrest and DNA repair or, if the damage is too great, initiate death via apoptosis. Cells lacking p53 accumulate damaged DNA and can become malignantly transformed (19, 20). We proposed that hypochlorous acid-dependent increases in p53 and WAF1/CIP1 could be important responses by which cells minimize malignant transformations and the carcinogenicity associated with inflammation.

We have now extended our earlier work and investigated whether hypochlorous acid arrests the growth of cultured human skin fibroblasts and if this is dependent on p53. Since chloramines are likely to be major products of hypochlorous acid at sites of inflammation and retain similar reactivity, we have determined whether they can also activate a p53–WAF1/CIP1 growth arrest pathway.

EXPERIMENTAL PROCEDURES

Cell culture and treatment. All reagents were from Sigma (Castle Hill, Australia) unless otherwise stated. Informed parental consent was obtained to isolate fibroblasts from human infant foreskins derived from routine circumcision. Fibroblasts were cultured as monolayers in minimum essential medium, 15% fetal calf serum, penicillin/streptomycin, and glutamine (all from Life Technologies, Auckland, New Zealand) in an incubator at 37°C and with an atmosphere of 5% CO_2. Cells were used between passages 4 and 10 and treated during the log phase of growth, when they were approximately 30% confluent. Cells were cultured in a variety of vessels dependent on the number required for each experiment. The media was removed, and cells were washed twice with 10 mM phosphate buffer, pH 7.4, containing 140 mM sodium chloride (PBS) and treated in Hanks' buffer (PBS containing glucose 1 mg ml^{-1}, magnesium 0.5 mM, and calcium 1 mM). Hypochlorous acid (Reckitt and Coleman, Auckland, New Zealand) was added as a bolus or as a flux generated by glucose oxidase and myeloperoxidase as described previously (17). Chloramines were synthesized by reacting glycine, ammonia, or taurine (5 mM) with hypochlorous acid (1 mM) and used immediately. The concentration of chloramines was determined using 5-thio-2-nitrobenzoic acid (21). The fivefold excess of amines was used to ensure that the hypochlorous acid reacted with the amines to form only monochloramines (11). As a consequence, solutions used to treat cells also contained four times as much amine as chloramines.

Growth arrest. Fibroblast growth was determined by measuring DNA synthesis and by counting numbers of cells. Fibroblasts were seeded in 96-well plates (approximately 1000 per well) and treated 48 h later, when there was approximately 2000 cells per well. After treatment, cells were washed twice with PBS and the reserved media was replaced. DNA synthesis was measured as the incorporation of BrdU using a cell proliferation kit following the manufacturer's instructions (Boehringer Mannheim, Auckland, New Zealand). Cell number was determined by counting the fibroblasts in a given area of the well and multiplying by total area of the well.

P53 and WAF1/CIP1 levels. After treatment with hypochlorous acid or chloramines in 8-cm plates, fibroblasts (approximately 1.5×10^6) were washed twice with PBS and scraped off with a rubber policeman. Nuclear and cytoplasmic extracts were prepared as described previously (17). Protein content of the extracts was determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, California). Equal amounts of protein extracted from cells that had been subjected to the various experimental conditions were separated on 10% polyacrylamide/sodium dodecyl sulfate gels and transferred to nitrocellulose membranes (Amersham, Little Chalfont, UK). Nuclear extracts were probed with anti-p53 antibody DO7 (Novacastra Laboratories, Newcastle upon Tyne, UK), and cytoplasmic extracts with anti-WAF1/CIP1 antibody (Zymed, San Francisco). A peroxidase-conjugated secondary antibody and 3,3′,5,5′-tetramethylbenzidine were used to visualize the immunoreactive proteins.

Cytotoxicity. Fibroblasts in 24-well plates (20×10^3 $well^{-1}$) were incubated with ^{51}Cr for 4 h in their media, washed, and then treated with reactive chlorine species in Hanks' buffer. Conditioned media was added back to the cells, and cell integrity was measured as the amount of ^{51}Cr released into the media over 24 h (17).

Inhibition of the transcriptional activating function of p53. A 20-mer oligonucleotide containing a consensus sequence for the p53–DNA binding site (5′-GGACATGCCCGGGCATGTCC-3′) was purchased from Canterbury Health Laboratories, Christchurch, New Zealand. A double-stranded oligonucleotide was annealed and preincubated with FuGene6 transfection reagent following the manufacturers instructions (Boehringer Mannheim, Auckland, New Zealand). As a control, a double-stranded oligonucleotide that contained the binding site for the Oct2A transcription factor, but not p53, was obtained (Boehringer Mannheim, Auckland, New Zealand) and preincubated with FuGene6. Fibroblasts in 96-well plates or 8-cm dishes were incubated with 12 ng or 2 μg, respectively, of p53-binding or non-p53-binding (Oct2A) oligonucleotide–FuGene6 mixture in media for 18 h prior to use. The effects of the transfected oligonucleotides on expression of WAF1/CIP1 and DNA synthesis were then determined as described above.

RESULTS

Effects of Reactive Chlorine Species of Viability of Cultured Human Skin Fibroblasts

Before determining the effects of hypochlorous acid and chloramines on growth arrest, it was important to demonstrate what concentrations of these reactive chlorine species could be used to induce a response without promoting cytotoxicity. Their effect on cell viability was determined by measuring the release of ^{51}Cr from treated fibroblasts (Fig. 1). Ammonia chloramine was nontoxic below 20 μM, whereas hypochlorous acid did not affect cell viability until its concentration exceeded 50 μM. Glycine chloramine and taurine chloramine were not toxic below 100 μM. Based on these results, cells were treated with 20 μM or less of the reactive chlorine species in all subsequent experiments. At these concentrations, hypochlorous acid or the chloramines did not promote apoptosis as measured by annexin V binding (results not shown).

To examine how rapidly hypochlorous acid and chloramines reacted with fibroblasts, we added 20 μM of these compounds to the cells and measured levels re-

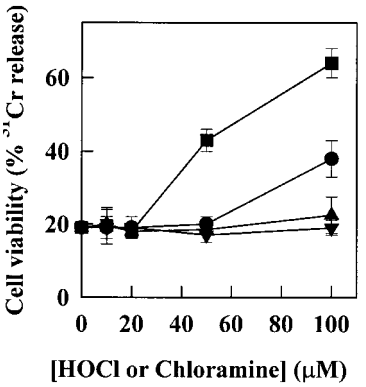

FIG. 1. Cytotoxicity of hypochlorous acid and chloramines. Cultured human skin fibroblasts were pretreated with ^{51}Cr and then treated with hypochlorous acid (●), ammonia chloramine (■), glycine chloramine (▲), or taurine chloramine (▼) for 1 h in Hanks' buffer. The amount of ^{51}Cr released into the media was measured 24 h later and expressed as a percentage of the total detergent releasable ^{51}Cr. The results shown are the means ± SD of one experiment done in triplicate and are typical of one other experiment.

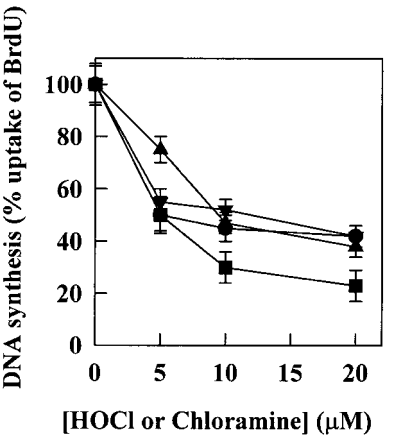

FIG. 3. Hypochlorous acid and chloramines inhibit DNA synthesis in cultured human skin cells. Cells were treated with hypochlorous acid (●), taurine chloramine (▼), glycine chloramine (▲), or ammonia chloramine (■) for 1 h in 100 μl Hanks' buffer. DNA synthesis was measured as the incorporation of BrdU immediately after treatment. Results shown are means ± SD of triplicates from one experiment and are typical of two others.

maining in the Hanks' buffer over a 1-h period. Both hypochlorous acid and ammonia chloramine were almost completely gone from the buffer within 10 min of addition (Fig. 2). In contrast more than 70% of the taurine and glycine chloramine remained 1 h after addition to the cells.

Hypochlorous Acid and Chloramines Cause Growth Arrest in Cultured Human Skin Fibroblasts

To gauge the effect of reactive chlorine species on growth arrest of cultured cells, fibroblasts were treated with either bolus additions of hypochlorous acid and chloramines or a flux of hypochlorous acid. Concentrations of hypochlorous acid as low as 5 μM inhibited DNA synthesis and maximal inhibition occurred at 10–20 μM (Fig 3). Similar effects resulted from treatment with taurine chloramine, glycine chloramine, or ammonia chloramine (Fig 3). Taurine, glycine, or ammonia added at concentrations of 40–80 μM did not inhibit DNA synthesis (data not shown). Fluxes of hypochlorous acid generated by glucose oxidase and myeloperoxidase, which mimic oxidant production by neutrophils, also inhibited DNA synthesis. A flux as low as 20 μM h^{-1} was sufficient to retard DNA synthesis (Fig 4).

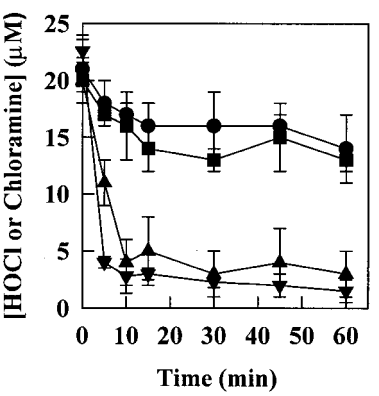

FIG. 2. Reactivity of hypochlorous acid and chloramines with cultured human skin fibroblasts. Hypochlorous acid (20 μM) (▼), ammonia chloramine (20 μM) (▲), glycine chloramine (20 μM) (●), or taurine chloramine (20 μM) (■) were added to cells in Hanks' buffer. Aliquots of buffer were removed at the intervals shown and the amount of hypochlorous acid and chloramine was measured using 5-thio-2-nitrobenzoic acid. The results shown are the means and ranges of one experiment done in duplicate and are typical of two other experiments.

Inhibition of DNA synthesis occurred within 1 h after exposing cells to 20 μM hypochlorous acid (Fig. 5). It continued for 24 h and eventually returned to normal 48 h later. Similarly, chloramines caused maximal inhibition of DNA synthesis 1 h after treatment and returned to normal after 2 days (data not shown).

In accord with the effect on DNA synthesis, low concentrations of hypochlorous acid and chloramines inhibited cell division (Fig 6). It is also evident from Fig. 6 that exposure to these concentrations of reactive chlorine species did not cause a loss of cells, which agrees with our data on cytotoxicity (Fig. 1). Inhibition of cell division was also reversible, and by 72 h after treatment the fibroblasts had recommenced dividing (Fig. 6). Glycine, taurine, and ammonia added at a concentration of 80 μM had no effect on cell division (data not shown).

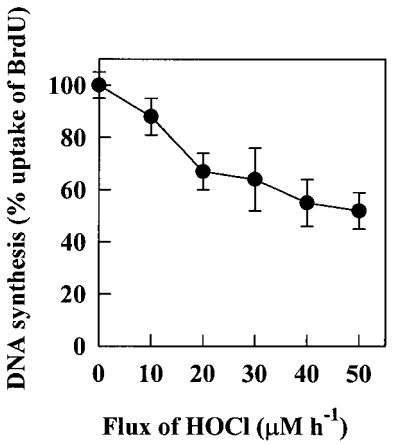

FIG. 4. Hypochlorous acid generated as a flux inhibits DNA synthesis of cultured human skin fibroblasts. Cells were exposed to a flux of hypochlorous acid generated by glucose oxidase (0–0.3 mg ml^{-1}) and myeloperoxidase (40 nM) for 1 h in 100 μl Hanks' buffer. DNA synthesis was measured at the end of 1 h as the incorporation of BrdU. The results shown are the means ± SD for three experiments.

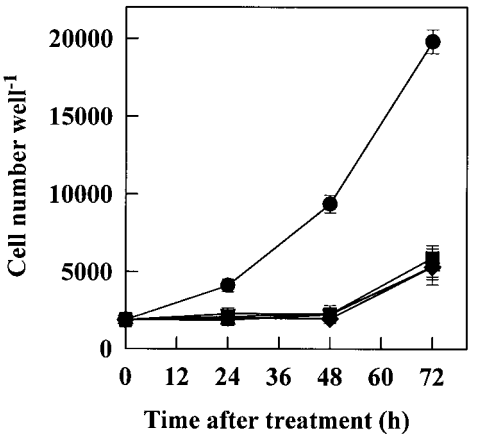

FIG. 6. Hypochlorous acid and chloramines arrest the division of fibroblasts. Cells were untreated (●) or treated with 20 μM hypochlorous acid (◆), 20 μM ammonia chloramine (■), 20 μM taurine chloramine (▼), or 20 μM glycine chloramine (▲) for 1 h in 100 μl Hanks' buffer. The medium was then replaced and the cells were counted at the time intervals shown. Results are given as the means ± SD of triplicates from one experiment and are typical of two others. In some cases the error bars lie within the symbol size.

Concentrations of Hypochlorous Acid and Chloramines That Initiate Growth Arrest Also Activate p53 and WAF1/CIP1

To establish whether or not the growth arrest caused by reactive chlorine species was mediated by p53 and WAF1/CIP1, we also determined the levels of these proteins in treated cells. Hypochlorous acid, taurine chloramine, glycine chloramine, or ammonia chloramine added as a 20 μM bolus increased the amount of immunoreactive p53 in nuclear extracts and WAF1/CIP1 in the cytoplasm 1 h after treatment of fibroblasts (Fig. 7). A flux of hypochlorous acid as low as 20 μM h^{-1} generated over 1 h was also effective at elevating p53 and WAF1/CIP1 (Fig. 7). Taurine or ammonia (80 μM) had no effect (Fig. 7) and neither did 80 μM glycine (result not shown). Levels of p53 and WAF1/CIP1 in the cytoplasmic and nuclear extracts, respectively, were unaltered by exposure of cells to hypochlorous acid or chloramines.

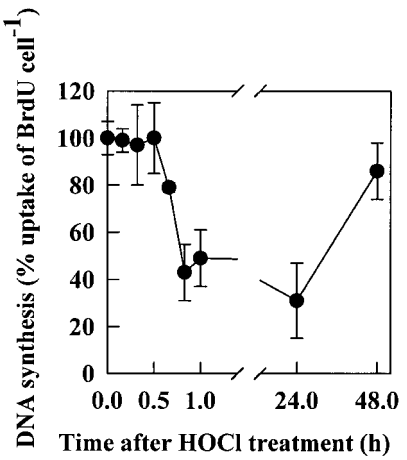

FIG. 5. Hypochlorous acid rapidly inhibits DNA synthesis. Cells were treated with hypochlorous acid (20 μM) in 100 μl Hanks' buffer for the time interval shown for times 1 h and less and for 1 h for other intervals. DNA synthesis was measured as the incorporation of BrdU. The results shown are the means ± SD for three experiments.

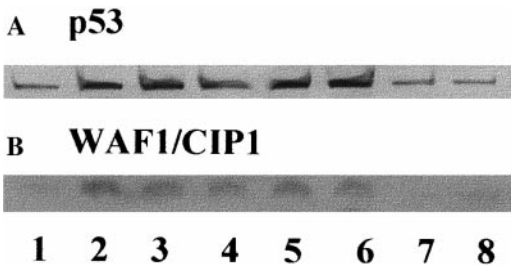

FIG. 7. Hypochlorous acid and chloramines increase p53 and WAF1/CIP1 in cultured human skin fibroblasts. Cells were (1) untreated or treated with (2) a bolus of 20 μM hypochlorous acid (3), a flux of hypochlorous acid (20 μM generated over 1 h with glucose oxidase (0.1 μg ml^{-1}) and myeloperoxidase (40 nM)), (4) 20 μM taurine chloramine, (5) 20 μM ammonia chloramine, (6) 20 μM glycine chloramine, (7) 80 μM taurine, or (8) 80 μM ammonia for 1 h in Hanks' buffer. Immediately after treatment cells were harvested and cytoplasmic and nuclear extracts were prepared. Extracts (nuclear, 30 μg; cytoplasmic, 60 μg) were electrophoresed and (A) p53 and (B) WAF1/CIP1 were detected in the nuclear and cytoplasmic extracts, respectively, by Western blot. The results shown are typical of three experiments.

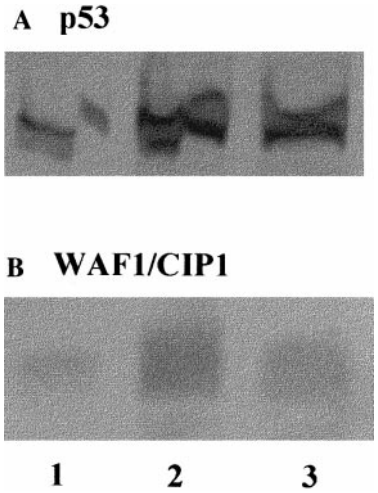

FIG. 8. A p53-binding oligonucleotide inhibits the activation of WAF1/CIP1 by hypochlorous acid. Cultured human skin fibroblasts were transfected overnight with an oligonucleotide containing a sequence that does not bind p53 (lane 2) or an oligonucleotide that does bind p53 (lane 3). Cells (1.5×10^6) were then left untreated (lane 1) or treated with hypochlorous acid (20 μM) for 1 h in Hanks' buffer (lanes 2 and 3). Immediately after treatment cells were harvested and cytoplasmic and nuclear extracts were prepared. Extracts (nuclear, 30 μg; cytoplasmic, 60 μg) were electrophoresed and (a) p53 and (b) WAF1/CIP1 were detected in the nuclear and cytoplasmic extracts, respectively, by Western blot. The results shown are typical of two experiments.

Growth Arrest Is Dependent on the Transcriptional Activation of WAF1/CIP1 by p53

The time course of the inhibition of DNA synthesis by hypochlorous acid (Fig. 5) and activation of p53 and WAF1/CIP1 (Fig. 7) is consistent with the possibility that the growth arrest was dependent on p53. To seek direct evidence for this, we transiently transfected cells with an oligonucleotide that binds the DNA-binding region of p53. In these cells, WAF1/CIP1 was not as elevated after treatment with hypochlorous acid compared to fibroblasts that had been transfected with an oligonucleotide that does not bind p53 (Fig. 8). Likewise, inhibition of DNA synthesis by hypochlorous acid was abolished only in fibroblasts that had been transfected with the p53-binding oligonucleotide (Fig. 9). These results demonstrate that growth arrest caused by reactive chlorine species was dependent on p53.

DISCUSSION

Previously we have shown that hypochlorous acid generated by neutrophils elevates levels of p53 and WAF1/CIP1 in cultured human fibroblasts (17). We have now extended this work to show that chloramines act similarly to hypochlorous acid and that all these reactive chlorine species retard DNA synthesis and cell division. Our findings that increases in WAF1/CIP1 and inhibition of DNA synthesis were substantially abrogated in cells transfected with an oligonucleotide that attaches to the DNA-binding region of p53 demonstrate that reactive chlorine species trigger a p53-dependent pathway that results in growth arrest. Under the conditions of our experiments, growth arrest was not a result of cytotoxicity because cells were exposed to sublethal concentrations of hypochlorous acid and chloramines that did not result in a decline in cell numbers. In this investigation, hypochlorous acid added as a bolus or a flux increased p53 and WAF1/CIP1 and caused growth arrest in fibroblasts within 1 h of treatment. Previously, we found that p53 was increased at 24 h after exposure of cells to a flux of hypochlorous acid but not with a bolus of this oxidant (17). It is apparent that the kinetics of the increase in p53 differ between the two methods of exposure to hypochlorous acid.

We found that low concentrations of chloramines and minimal fluxes of hypochlorous acid caused elevation of p53, inhibition of DNA synthesis, and growth arrest. In combination, these results highlight how exquisitely sensitive the p53-dependent growth arrest pathway is to reactive chlorine species. Its sensitivity to hypochlorous acid and chloramines indicates that this pathway is very likely to be switched on in bystander cells when neutrophils are stimulated at sites of inflammation. Furthermore, reactive chlorine species may be responsible for the increased levels of p53 that are seen in

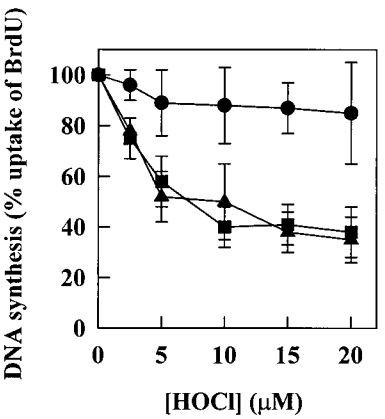

FIG. 9. Inhibition of the transcriptional activating function of p53 prevents hypochlorous acid-dependent growth arrest. Cultured human skin fibroblasts were transfected overnight with a p53-binding oligonucleotide (●) or an oligonucleotide containing a sequence that does not bind p53 (▲) or they were left untransfected (■). Cells were then treated with increasing concentrations of hypochlorous acid for 1 h in 100 μl Hanks' buffer. Immediately after treatment DNA synthesis was measured as the incorporation of BrdU. The results shown are the means ± SD of three experiments.

cells surrounding sites of inflammation (22–26). Our results imply that reactive chlorine species have the potential to act on various signaling pathways in the inflammatory response. There is accumulating support for this proposal. In recent work from our laboratory, it was shown that sublethal concentrations of hypochlorous acid initiated apoptosis and growth arrest in cultured endothelial cells (27). Others have shown that hypochlorous acid induces the expression of apurinic endonuclease (also known as Ref-1) in Chinese-hamster ovary cells (28) and activates NF-κB in a T-lymphocyte cell line, presumably through secondary chloramines formed in media (29). Thus, hypochlorous acid and chloramines should no longer be thought of as solely cytotoxic oxidants. Rather, more consideration should be given to their subtle reactivities that modulate cellular events. Their unique chemistry undoubtedly explains why a concentration of hydrogen peroxide almost 10-fold higher than that of hypochlorous acid is required to achieve an equivalent increase in p53 and WAF1/CIP1 (17). Thiols and thioethers are most susceptible to oxidation by reactive chlorine species (10). Therefore, they are the most likely targets when cells are treated with sublethal doses of hypochlorous acid or chloramines. Intracellular thiol groups differ in their reactivity with hypochlorous acid (30), so it is conceivable that particular thiol-containing proteins may be regulated by reactive chlorine species.

The differing reactivities of hypochlorous acid and chloramines will influence the extent to which they initiate growth arrest in the physiological milieu. Hypochlorous acid is an indiscriminant oxidant that reacts rapidly with numerous biomolecules (9, 31) whereas chloramines are less reactive and more selective in the type of reactions they undergo (11). This is apparent from our result that hypochlorous acid and ammonia chloramine decayed rapidly upon addition to fibroblasts but about 75% of the taurine chloramine and glycine chloramine remained after an hour (Fig. 2). Other chloramines such as those of α amino acids have lifetimes intermediate between ammonia chloramine and taurine chloramine and breakdown to aldehydes (32). Given that all the chloramines were as effective as hypochlorous acid at inhibiting DNA synthesis and cell division, it is evident that chloramines that have longer half-lifes at sites of inflammation should have a greater impact on growth arrest.

Our findings indicate that reactive chlorine species produced at sites of inflammation will counteract the mutagenic effects of neutrophils. The effects of these oxidants should allow for cellular repair since inhibition of DNA synthesis was maintained for at least 24 h while growth arrest continued for 48 h. Thus, by initiating a p53-dependent pathway to growth arrest, they will enable bystander cells to minimize the possibility of malignant transformations. This situation will change when p53 is mutated so that it can not activate WAF1/CIP1 (19). With mutated p53, the mutagenic effects of chloramines and other oxidants would go unchecked, resulting in the eventual development of cancers.

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