Phagocytes Produce 5-Chlorouracil and 5-Bromouracil, Two Mutagenic Products of Myeloperoxidase, in Human Inflammatory Tissue

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Abbreviations: DTPA, diethylenetriaminepentaacetic acid; GC, gas chromatography; M⁺, molecular ion; MS, mass spectrometry; m/z, mass-to-charge ratio; TMS, trimethylsilyl; PFB, pentafluorobenzyl; PMA, phorbol myristate acetate.

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Abstract

Oxidative damage to DNA has been implicated in carcinogenesis during chronic inflammation. Epidemiological and biochemical studies suggest that one potential mechanism involves myeloperoxidase, a heme protein secreted by human phagocytes. In this study, we demonstrate that human neutrophils use myeloperoxidase to oxidize uracil to 5-chlorouracil in vitro. Uracil chlorination by myeloperoxidase or reagent HOCl exhibited an unusual pH dependence, being minimal around pH 5 but increasing markedly under either acidic or mildly basic conditions. This bimodal curve suggests that myeloperoxidase initially produces HOCl, which subsequently chlorinates uracil by acid- or base-catalyzed reactions. Human neutrophils use myeloperoxidase and H$_2$O$_2$ to chlorinate uracil, suggesting that nucleobase halogenation reactions may be physiologically relevant. Using a sensitive and specific mass spectrometric method, we detected two products of myeloperoxidase—5-chlorouracil and 5-bromouracil—in neutrophil-rich human inflammatory tissue. Myeloperoxidase is the most likely source of 5-chlorouracil in vivo because the halogenated uracil is a specific product of the myeloperoxidase system in vitro. In contrast, previous studies have demonstrated that 5-bromouracil could be generated by either eosinophil peroxidase or myeloperoxidase, which preferentially brominates uracil at plasma concentrations of halide and under moderately acidic conditions. These observations indicate that the myeloperoxidase system promotes nucleobase halogenation in vivo. Because 5-chlorouracil and 5-bromouracil can be incorporated into nuclear DNA, and these thymine analogs are well-known mutagens, our observations raise the possibility that halogenation reactions initiated by phagocytes provide one pathway for mutagenesis and cytotoxicity at sites of inflammation.
Introduction

Chronic inflammation is implicated in the pathogenesis of human cancer, and many lines of evidence point to oxidative modification of DNA as one potential mechanism (1,2). Phagocytic white blood cells, the cellular hallmark of inflammation, possess an NADPH oxidase that is a well-characterized source of reactive intermediates that damage DNA (3,4). The enzyme’s initial product is superoxide, which dismutates to hydrogen peroxide ($\text{H}_2\text{O}_2$). The oxidizing equivalents in $\text{H}_2\text{O}_2$ are used by myeloperoxidase (5) (6,7), a heme protein expressed by neutrophils, monocytes, and macrophages (8) (9). At plasma halide concentrations, the major activity of myeloperoxidase is oxidation of chloride to hypochlorous acid (HOCl) (10,11).

$$\text{Cl}^- + \text{H}_2\text{O}_2 + \text{H}^+ \rightarrow \text{HOCl} + \text{H}_2\text{O} \quad \text{(Equation 1)}$$

This HOCl is in equilibrium with molecular chlorine ($\text{Cl}_2$).

$$\text{HOCl} + \text{H}^+ + \text{Cl}^- \rightleftharpoons \text{Cl}_2 + \text{H}_2\text{O} \quad \text{(Equation 2)}$$

Eosinophil peroxidase, an enzyme closely related to myeloperoxidase, produces hypobromous acid (HOBr) (12). HOCl produced by myeloperoxidase will also generate brominating oxidants \textit{in vitro} and \textit{in vivo} by a pathway that might involve bromine chloride (BrCl), HOBr or other halogenating intermediates (13).

$$\text{HOCl} + \text{H}^+ + \text{Br}^- \rightleftharpoons \text{BrCl} + \text{H}_2\text{O} \rightleftharpoons \text{HOBr} + \text{H}^+ + \text{Cl}^- \quad \text{(Equation 3)}$$

HOCl is a potent cytotoxin that plays key roles in host defense by oxidizing cellular constituents of invading pathogens (5)(6,7). This reactive species also has the potential to damage proteins and lipids in host tissue (14-17).

By damaging DNA and other constituents of host cells, generation of halogenating oxidants by myeloperoxidase might contribute to the association between chronic inflammation and cancer (13,18,19)[Masuda, 2001 #461]. Indeed, a polymorphism in the myeloperoxidase
promoter that increases the enzyme’s expression in vitro is strongly associated with increased risk of leukemia, lung cancer, and aerodigestive tract cancers (20-25).

In the current studies, we have investigated the role of myeloperoxidase in chlorinating uracil in vitro and used isotope dilution gas chromatography/mass spectrometry to identify 5-chlorouracil and 5-bromouracil in neutrophil-rich human inflammatory tissue. Our observations indicate that halogenating intermediates generated by phagocyte peroxidases represent one potential pathway for modifying nucleic acids at sites of inflammation in vivo.
Experimental Procedures

**Materials.** Myeloperoxidase (donor: hydrogen peroxide, oxidoreductase, EC 1.11.1.7) was isolated from HL60 cells by sequential lectin affinity and size exclusion chromatography (26,27). Enzyme concentration (A$_{430}$/A$_{280}$ > 0.8) was determined spectrophotometrically ($\varepsilon_{430} = 178$ mM$^{-1}$cm$^{-1}$; (28)). Chloride-free sodium hypochlorite (NaOCl) was prepared as described (18,29). The concentration of NaOCl was determined spectrophotometrically ($\varepsilon_{292} = 350$ M$^{-1}$cm$^{-1}$; (30)). 5-[$^{13}$C$_4$,^{15}N$_2$]Bromouracil was synthesized as described (19). For isotope-labeled 5-chlorouracil, [$^{13}$C$_4$,^{15}N$_2$]uracil (1 mM; Cambridge Isotope Laboratories, Inc., Andover, MA) was exposed to 1 mM HOCl for 15 min at 25$^\circ$C in 50 mM phosphoric acid and 1 M NaCl. The reaction was quenched by adding 6 mM L-methionine. 5-[$^{13}$C$_4$,^{15}N$_2$]Chlorouracil (isotope enrichment > 98%) was isolated by HPLC. The synthetic compound was identical to authentic 5-chlorouracil by HPLC and GC retention times; its TMS derivative exhibited an identical electron ionization mass spectrum with major fragment masses increased by 6 amu.

**Uracil oxidation.** Reactions were performed in buffer A (100 mM sodium chloride, 100 $\mu$M DTPA, 50 mM sodium phosphate, pH 7). They were initiated by adding oxidant (H$_2$O$_2$ or HOCl) to vortexed samples and terminated by adding 6 mM L-methionine. The concentration of H$_2$O$_2$ was determined spectrophotometrically ($\varepsilon_{240} = 39.4$ M$^{-1}$cm$^{-1}$; (31)). The pH of the reaction mixtures was varied using phosphoric acid and Chelex-100 (BioRad)-treated monobasic sodium phosphate and dibasic sodium phosphate.

**Human neutrophils.** Neutrophils were prepared by density gradient centrifugation (32) and suspended in buffer B (10 mM sodium phosphate, 100 mM NaCl, 1.4 mM CaCl$_2$, 1.4 mM MgSO$_4$, 2 mM dextrose, 100 $\mu$M DTPA, pH 7) supplemented with 1 mM uracil. At the end of the incubation, the supernatant was extracted with 2-propanol/diethyl ether (22:78, v/v) (33).
which was then back-extracted with 0.2 M ammonium hydroxide and concentrated to dryness under vacuum. The extract was derivatized for electron capture gas chromatography mass spectrometry as described below.

**Reverse-phase HPLC.** Uracil oxidation products were analyzed and purified for NMR analysis as described (34). For mass spectrometric analysis, HPLC fractions were collected and concentrated under nitrogen. Uracils extracted from human tissue were eluted from an HPLC reverse-phase column (34) at 1 mL/min with 95% solvent A (50 mM ammonium acetate) and 5% solvent B (50 mM ammonium acetate in 90% methanol) for 4 min followed by a linear gradient to 100% solvent B over 20 min.

**NMR.** HPLC-purified reaction product was solubilized in D$_2$O and analyzed at 25 °C with a Varian Unity-Plus 500 spectrometer (499.843 MHz for $^1$H) equipped with a Nalorac indirect detection probe. $^1$H chemical shifts were referenced to external sodium 3-(trimethylsilyl)propionate-2,2,3,3-$d_4$ in D$_2$O. Spectra were recorded from 8 transients with a 12 s preacquisition delay over a spectral width of 8000 Hz.

**Uracil extraction from tissue.** The Human Studies Committee at Washington University School of Medicine in St. Louis approved all protocols. L-Methionine (6 mM final concentration) was added to tissue obtained for clinical indications and immediately frozen at -80°C. Human plasma and urine were obtained from healthy volunteers. Following the addition of isotope-labeled internal standards ([$^{13}$C$_4$, $^{15}$N$_2$]-labeled uracil, 5-chlorouracil, and 5-bromouracil, and [$^{13}$C$_2$, $^{15}$N]uracil for monitoring artifactual halogenation), the thawed samples were extracted with 2-propanol/diethylether (22:78, v/v) (33), which was back-extracted with 0.2 M ammonium hydroxide and then fractionated by reverse-phase HPLC as described above.
Buffer A supplemented with uracil was subjected to the same isolation procedure and analyzed to ensure that ex vivo oxidation or contamination did not account for any observed analyte.

*Gas chromatography-mass spectrometry (GC/MS)*. Pentafluorobenzyl derivatives of pyrimidines were quantified by isotope dilution GC/MS [Heinecke, 1999 #460]. For tissue samples analysis, HPLC fractions were suspended in 0.2 M sodium phosphate (pH 10.5) containing 0.5 M tetrabutylammonium hydrogen sulfate. Uracil, 5-chlorouracil, and 5-bromouracil were alkylated for 60 min at 25°C with dichloromethane containing pentafluorobenzyl bromide (33). Following centrifugation, the aqueous phase was removed, and the organic phase was dried under nitrogen. Residual derivatizing agent was removed by silica gel chromatography (35). The derivatives were suspended in toluene, and 1 μL aliquots were injected using an on-column inlet on a Hewlett-Packard 6890 gas chromatograph equipped with a 12 m DB-1701 capillary column (0.2 mm i.d., 0.33 μm film thickness; J&W Scientific) interfaced with a Hewlett-Packard 5973 mass spectrometer. Ions were monitored in the negative ion electron capture ionization mode with methane as the reagent gas. Injector and interface temperatures were 280°C and 290°C, respectively. The initial GC oven temperature was 170°C for 1 min, followed by a 10°C/min increasing ramp to 250°C. Toluene was analyzed between samples to ensure that traces of analyte were not being carried over. TMS derivatives of pyrimidines were prepared and analyzed by GC/MS as described (18).
Results

The myeloperoxidase-H$_2$O$_2$-Cl$^-$ system chlorinates uracil at plasma concentrations of halide ions. We exposed uracil to the myeloperoxidase-H$_2$O$_2$-Cl$^-$ system in buffer A (100 mM sodium chloride, 100 µM DTPA, 50 mM sodium phosphate, pH 7) and used HPLC to characterize the product. The reaction mixture yielded a new peak of material with a retention time and UV/visible absorption spectrum identical to that of authentic 5-chlorouracil (Fig. 1A,B). The structure of the uracil oxidation product was investigated by GC/MS (Fig. 1C). The GC retention times and electron ionization mass spectra of the TMS derivatives of the oxidation product and authentic 5-chlorouracil were essentially identical. The oxidation product exhibited a molecular ion at $m/z$ 290 and a prominent fragment ion at $m/z$ 275 consistent with the $^\cdot$CH$_3$ loss typical of TMS derivatives. These ions also exhibited prominent (M + 2) isotope peaks consistent with the isotopic abundance of $^{35}$Cl and $^{37}$Cl, strongly suggesting a monochlorinated product. Ions consistent with loss of chlorine radical were observed at $m/z$ 255 ([M - Cl]$^+$ and $m/z$ 239 ([M - CH$_3$ - HCl]$^+$ or [M - CH$_4$ - Cl]$^+$). As anticipated for compounds that lack chlorine, these fragment ions no longer exhibited the prominent (M + 2) isotopic pattern.

Myeloperoxidase chlorinates uracil at C-5 of the pyrimidine ring. To determine the position of chlorine on the pyrimidine ring of uracil, we used HPLC to isolate the product from the myeloperoxidase reaction mixture and analyzed it with high-resolution $^1$H NMR. The spectrum of the product was essentially identical to that of authentic 5-chlorouracil (data not shown), but it was distinct from that of 6-chlorouracil. Significant features included loss of the C-5 proton resonance, a downfield shift in the C-6 proton, and conversion of the C-6 proton resonance from a doublet to a singlet. These findings are consistent with substitution of a chlorine atom at the C-5 position of the pyrimidine ring.
5-Chlorouracil is a specific product of myeloperoxidase. We used reverse-phase HPLC to characterize uracil chlorination by various peroxidases. 5-Chlorouracil was generated by myeloperoxidase but not by eosinophil peroxidase, lactoperoxidase, or horseradish peroxidase. The reaction required myeloperoxidase, H₂O₂, and Cl⁻. It was blocked by catalase, a scavenger of H₂O₂, and azide and 3-aminotriazole, two inhibitors of heme enzymes. Methionine, a potent scavenger of HOCl, also blocked the reaction (Table 1). These results demonstrate that chlorination of uracil by myeloperoxidase requires active enzyme, Cl⁻, and H₂O₂. Adding 4-hydroxyphenylacetic acid, a phenolic compound, caused only a minor reduction in 5-chlorouracil yield (36). This observation suggests that myeloperoxidase chlorinates uracil more readily than it chlorinates a phenolic ring.

Reagent HOCl generated 5-chlorouracil almost quantitatively at neutral pH in buffer A, which contained a plasma concentration of NaCl. In contrast, two different systems that generate hydroxyl radical—FeSO₄ plus excess H₂O₂ and CuSO₄ plus excess H₂O₂ (37)—failed to chlorinate uracil, as did FeSO₄ alone or glucose (Table 1). Collectively, these results indicate that HOCl generated by myeloperoxidase converts uracil into 5-chlorouracil and that hydroxyl radical, ferrous iron, or glycoxidation reactions are unable to chlorinate uracil under our experimental conditions.

Ascorbate and thiols potently inhibit uracil chlorination by myeloperoxidase. We used HPLC to examine the effects of antioxidants on the yield of 5-chlorouracil produced by the myeloperoxidase system (Table 2). The water-soluble antioxidants ascorbate, N-acetyl cysteine, and glutathione, which react with HOCl to respectively form dehydroascorbate and oxygenated thiols, inhibited uracil chlorination almost completely. Taurine, which reacts with HOCl to produce chloramines, significantly reduced the yield of 5-chlorouracil. Nitrite, a nitric oxide
breakdown product, also inhibited uracil chlorination, perhaps by acting as an alternative substrate for myeloperoxidase or by reacting with HOCl to produce nitrosyl chloride and nitrate. Less effective were trolox, a water-soluble vitamin E analog, and phenol, which would be expected to form $p$-chlorophenol. Mannitol, frequently used as a hydroxyl radical scavenger, had virtually no effect on 5-chlorouracil production. These observations indicate that water-soluble antioxidants that scavenge HOCl are effective inhibitors of uracil chlorination by myeloperoxidase.

_Uracil chlorination by myeloperoxidase has both acidic and neutral pH optima._ Uracil chlorination by myeloperoxidase at neutral pH increased with increasing substrate concentration and reaction time (Fig. 2) and was optimal at plasma concentrations of Cl$^{-}$ (100 mM). The product yield of 5-chlorouracil was $\sim$20% relative to peroxide. The pH dependence for chlorination by myeloperoxidase showed an unusual bimodality, with optima at pH 4 and pH 7 (Fig. 2). These observations indicate that myeloperoxidase produces high yields of 5-chlorouracil under both neutral and acidic conditions.

_Two distinct reaction pathways chlorinate uracil._ To better understand the mechanism for 5-chlorouracil production, we examined the pH dependence of 5-chlorouracil formation by HOCl, the chlorinating intermediate generated by myeloperoxidase (Fig. 3). Reagent HOCl produced quantitative yields of 5-chlorouracil under both strongly acidic and mildly basic conditions, with the product yield being lowest at pH 5. Because Cl$_2$ is in equilibrium with HOCl by a reaction requiring Cl$^{-}$ and H$^+$ (38), we explored its role in the reaction using chloride-free HOCl. Omitting NaCl (Fig. 3; - NaCl) had little effect on 5-chlorouracil yield when the reaction mixture was $> \text{pH 5}$. In contrast, omission of Cl$^{-}$ markedly diminished product yield when the pH was $< 5$. Under strongly acidic conditions, chlorination of uracil was independent of Cl$^{-}$, possibly
due to generation of Cl₂ from trace amounts of Cl⁻ contaminant.

At plasma concentrations of Cl⁻, chlorination of uracil was directly proportional to the concentration of HOCl in the reaction mixture at both pH 4 and pH 8 (Fig. 3). However, the yield of 5-chlorouracil was increased markedly by Cl⁻ at pH 4 but was independent of Cl⁻ at pH 8. The rate of uracil chlorination was also significantly increased by 100 mM Cl⁻ at pH 4 but not at pH 8 (Fig. 3). It is noteworthy that the reaction rate was highest at pH 8. In contrast, ring chlorination of deoxycytidine by HOCl is optimal under acidic conditions (18). These observations suggest that chlorination of uracil by myeloperoxidase involves two different reaction pathways. Under acidic conditions, a Cl₂-like species mediates chlorination. Under neutral conditions, chlorination involves HOCl or its conjugate base, ClO⁻ (pKₐ ~ 7.2).

Activated human neutrophils generate 5-chlorouracil. To determine whether human phagocytes can also chlorinate uracil, we incubated neutrophils in a physiological salt solution supplemented with 1 mM uracil. Phorbol ester-stimulated neutrophils produced a peak of material that co-migrated with 5-chlorouracil on HPLC. We isolated this material by HPLC, converted it to the pentafluorobenzyl derivative, and analyzed the derivative by negative ion chemical ionization GC/MS. The GC retention time, mass spectrum, and isotope pattern of the derivative identified the material as 5-chlorouracil. Formation of 5-chlorouracil required activation of the neutrophils with phorbol ester and was inhibited by azide and catalase, implicating a peroxidase and H₂O₂ in the reaction (Table 3). In contrast, superoxide dismutase modestly enhanced the product yield, perhaps by increasing the availability of H₂O₂ or by preventing superoxide from inactivating myeloperoxidase (39).

Human inflammatory tissue contains 5-chlorouracil and 5-bromouracil. To determine whether phagocytes halogenate uracil in vivo, we searched for 5-chlorouracil and 5-bromouracil
in tissue harvested from six humans suffering from a variety of bacterial infections. Each tissue sample represented an inflammatory exudate collected from a site of superficial infection and was rich in neutrophils and cellular debris. After adding internal standards and 6 mM L-methionine (a scavenger of HOCl and H₂O₂) to the samples, uracils were extracted and subjected to reverse-phase HPLC. Materials eluting with the HPLC retention time of uracil, 5-chlorouracil, or 5-bromouracil were converted to their pentafluorobenzyl derivatives and analyzed with negative ion electron capture GC/MS.

The samples of inflammatory tissue contained 60-600 µM uracil, a substrate for halogenation by myeloperoxidase. GC/MS analysis revealed two peaks of material that exhibited major ions and retention times identical to those of authentic 5-chlorouracil and 5-bromouracil (Fig. 4). Selected ion monitoring demonstrated that the ions derived from 5-chlorouracil and 5-bromouracil co-eluted with those derived from 5-chloro[¹³C₄,¹⁵N₂]uracil and 5-bromo[¹³C₄,¹⁵N₂]uracil, respectively. The isotopic distribution of $^{35}$Cl and $^{37}$Cl was exploited to confirm the identity of 5-chlorouracil. Selected ion monitoring demonstrated the peak area ratio (∼3:1) of the ions at $m/z$ 325 and $m/z$ 327 expected for a monochlorinated compound. Similarly, the isotopic distribution of $^{79}$Br and $^{81}$Br demonstrated the peak area ratio (∼1:1) of the ions at $m/z$ 369 and $m/z$ 371 expected for a monobrominated compound. The mass-to-charge ratio of ions, isotopic patterns, and retention times on gas chromatography were consistent with the presence of endogenous 5-chlorouracil and 5-bromouracil in the human inflammatory tissue samples.

All of the infected patients had 5-chlorouracil and/or 5-bromouracil in their inflammatory tissue. Quantification by isotope dilution GC/MS demonstrated that 5-chlorouracil was present in samples from all six individuals with levels ranging from 0.7 to 36 µmol/mol uracil. Three of these samples also contained 5-bromouracil; levels of this halogenated
nucleobase ranged from 3.9 to 59 μmol/mol uracil. When 5-bromouracil was detected, it was more abundant than 5-chlorouracil, suggesting that eosinophil peroxidase contributed to uracil halogenation or that Br⁻ was present at high enough levels to favor bromination by myeloperoxidase (13,40).

To determine whether the 5-chlorouracil or 5-bromouracil detected in the samples might have been generated artifactually during workup, we performed three sets of experiments. First, the analytic procedure was applied to samples of plasma or urine from healthy donors. Under these conditions, neither halogenated base was detectable. Second, we quantified the levels of halogenated nucleobases in liver harvested from apparently healthy mice. We, again, failed to detect either 5-chlorouracil or 5-bromouracil (limit of detection, ~ 0.9 nmol/mol uracil) providing strong evidence that artifactual halogenation of uracil was not taking place during tissue work-up or analysis. Third, uracil labeled with two ¹³C atoms and one ¹⁵N atom was added to inflamed tissue. The material was extracted, derivatized, and analyzed by negative ion electron capture GC/MS (Fig. 4A,C). Any uracil that was artifactually chlorinated or brominated during the analysis would have been detected as halogenated [¹³C₂,¹⁵N₁]uracil. The base peaks (M – PFB⁻) of the pentafluorobenzyl derivatives of chlorinated or brominated [¹³C₂,¹⁵N₁]uracil have a m/z of 328 (³⁵Cl isotopomer) or 374 (⁸¹Br isotopomer), respectively. However, ions of m/z of 328 or 374 could also be attributed to the naturally occurring ¹³C present in endogenous 5-chlorouracil or 5-bromouracil. When a correction was made for the contribution of ¹³C derived from endogenous 5-chlorouracil (relative abundance of ¹³C to ¹²C is ~ 0.01), the peak of material with m/z 328 that coeluted with the 5-chlorouracil internal standard corresponded to a level of artifact < 1%. Similarly, peaks of material observed at m/z 374 accounted for only 1.6%, 1.7% and 5.2% of the endogenous 5-bromouracil observed in three independent analyses. Collectively,
these observations indicate that 5-chlorouracil and 5-bromouracil were present in inflamed human tissue and that the halogenated pyrimidines were not generated \textit{ex vivo} during extraction or analysis.
Discussion

Our results demonstrate that both purified myeloperoxidase and human neutrophils can chlorinate uracil \textit{in vitro}, and that inflamed human tissue contains both chlorinated and brominated uracil. These findings provide strong evidence that nucleobases are targeted for halogenation during inflammation in human tissue, raising the possibility that oxidants generated by myeloperoxidase and eosinophil peroxidase play key roles in the pathways for DNA damage during inflammation.

Myeloperoxidase is the most likely source of 5-chlorouracil \textit{in vivo} because the latter is a specific product of the myeloperoxidase system \textit{in vitro}. In contrast, 5-bromouracil could be generated by either eosinophil peroxidase or myeloperoxidase (13,34), both of which preferentially brominate uracil at plasma concentrations of halide (100 mM Cl\textsuperscript{−}, 100 µM Br\textsuperscript{−}). In fact, we have recently shown that 3-chlorotyrosine and 3-bromotyrosine production is inhibited in mice deficient in myeloperoxidase, indicating that the enzyme promotes both chlorination and bromination reactions \textit{in vivo} (40). 5-Chlorouracil and 5-bromouracil might also be generated indirectly by deamination of cytosine nucleosides that have been halogenated by phagocyte peroxidases (13,18).

Although only low concentrations of uracil, a substrate for myeloperoxidase halogenation, are normally present in plasma, the dissolution of dead cells that occurs during inflammation would presumably liberate nucleobases and nucleosides into the extracellular environment. Indeed, we found the uracil content of inflamed tissue fluid (as high as 600 µM) to be nearly 1000-fold higher than that of plasma, as assessed by isotope dilution GC/MS. Therefore, inflammation greatly increases the free pyrimidine content of tissue, providing myeloperoxidase with ample substrate for halogenation. The inflammatory milieu thus provides
all of the necessary factors—numerous phagocytes and a high local concentration of substrate—for halogenation of pyrimidines. Furthermore, since inflammation also causes cells to proliferate, it sets the stage for thymine analogs such as halogenated nucleobases to be incorporated into newly synthesized DNA (1).

The chlorination of uracil by myeloperoxidase or reagent HOCl exhibited an unusual pH dependence, being minimal around pH 5 but increasing markedly under either acidic or mildly basic conditions. This bimodal curve suggests that myeloperoxidase initially produces HOCl, which subsequently chlorinates uracil by acid- or base-catalyzed reactions. 5-Chlorouracil formation was independent of Cl⁻ under neutral and basic conditions, suggesting that chlorination involves HOCl. The increased reactivity of uracil at basic pH may derive from preferential halogenation of uracil anion (pKₐ ~ 9.5) or a different, tautomeric form of uracil. In contrast, chlorination by HOCl below pH 5 was strongly dependent on Cl⁻, suggesting the involvement of a Cl₂-like species in a different reaction pathway. These observations indicate that myeloperoxidase chlorinates uracil by two different pathways that appear to involve HOCl under basic conditions and Cl₂ under acidic conditions.

The precise role of antioxidants in preventing DNA damage in vivo will depend critically on the underlying chemistry for damage. Our in vitro experiments showed that the chlorination of uracil by HOCl was strongly inhibited by ascorbate and low molecular weight thiols. In contrast, the vitamin E analogue trolox and phenol had little effect. These results suggest that ascorbate and glutathione, but not phenolic compounds such as vitamin E, might help protect nucleobases from oxidative damage by halogenating intermediates in vivo. It is noteworthy that ascorbate promotes modification of nucleobases by products of lipid peroxidation in vitro (41), emphasizing the importance of understanding the chemical mechanisms of DNA damage in vivo.
Previous studies have demonstrated that the artifactual generation of halogenated and nitrated oxidation products is a major problem during the analysis of biological material (42-47). Indeed, some studies have found that virtually all of the oxidation products detected in tissue were generated artifactualy (46,47). This is particularly a problem when acidic conditions are used for sample hydrolysis or derivatization. However, we were unable to detect halogenated nucleobases in human plasma or urine, or in liver harvested from apparently healthy mice. Moreover, we directly monitored the \textit{ex vivo} formation of halogenated nucleobases by adding \textsuperscript{[13}C\textsubscript{2},\textsuperscript{15}N\textsubscript{1}]uracil to tissue samples and then monitoring the appearance of halogenated \textsuperscript{[13}C\textsubscript{2},\textsuperscript{15}N\textsubscript{1}]uracil. Artifactual halogenation accounted for <1\% of 5-chlorouracil and <5\% of 5-bromouracil that we detected in inflammatory tissue. Collectively, these observations provide strong evidence that 5-chlorouracil and 5-bromouracil were present in inflamed human tissue and that the halogenated pyrimidines were not generated \textit{ex vivo} during extraction or analysis.

While many studies have focused on direct oxidation of chromosomal DNA as a mechanism for mutagenesis (1,2), we recently showed that myeloperoxidase and eosinophil peroxidase brominate the free nucleobase uracil and the free nucleoside deoxycytidine \textit{in vitro} at plasma concentrations of chloride and bromide (13,19,34). Mass spectrometry, flow cytometric analysis, and immunohistochemical studies revealed that 5-bromouracil was incorporated into the DNA of dividing mammalian cells exposed to purified 5-bromodeoxycytidine produced by eosinophil peroxidase (19). Previous studies have also shown that 5-chlorouracil and 5-bromouracil can be taken up by mammalian cells and tissues and subsequently converted to their corresponding deoxynucleosides by thymidine phosphorylase (48,49). DNA polymerase then incorporates the resulting chlorodeoxyuridine and bromodeoxyuridine into DNA (50,51). Thus our detection of halogenated uracil may be significant, as 5-chlorodeoxyuridine and 5-
bromodeoxyuridine are well-established thymidine analog mutagens that mispair with guanine, causing GC to AT and AT to GC transitions (52). Based on our own observations and previous findings, we propose that nucleobases are halogenated by phagocytes and incorporated into host DNA during replication or repair in a process we term nucleotide precursor mutagenesis. In this way DNA may be damaged both indirectly by incorporation of halogenated nucleosides and directly by oxidation of DNA in situ.

We have demonstrated that 5-chlorouracil is a specific product of myeloperoxidase, and have detected two products of myeloperoxidase—5-chlorouracil and 5-bromouracil—in neutrophil-rich inflammatory tissue from humans. These observations indicate that human neutrophils use myeloperoxidase to halogenate uracil in vivo. Transition mutations occurring in genes containing these halogenated nucleobases could then alter cell cycle regulation, DNA replication, or DNA repair, creating a link between myeloperoxidase activity and increased cancer risk.
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Figure Legends

Figure 1. Reverse-phase HPLC analysis (A), UV absorption spectrum (B), and mass spectrum (C) of uracil oxidized by the myeloperoxidase-H$_2$O$_2$-Cl$^-$ system. (A) Uracil was incubated with the complete myeloperoxidase system (Complete; myeloperoxidase and H$_2$O$_2$). Where indicated, enzyme was omitted from the reaction mixture (-MPO) and 10 µM authentic 5-chlorouracil was included (-MPO, +5-chlorouracil). (B) UV/visible absorption spectra of the material with a retention time of 13 min. (C) Electron ionization mass spectrum of HPLC-purified, TMS-derivatized 5-chlorouracil generated by myeloperoxidase.

Figure 2. Reaction requirements for 5-chlorouracil generation by myeloperoxidase. Uracil was modified by incubation with the complete myeloperoxidase system in buffer A at pH 7 for 60 min at 37 °C. Where indicated, the H$_2$O$_2$ concentration (A), Cl$^-$ concentration (B), pH (C), and reaction time (D) were varied. 5-Chlorouracil was quantified by HPLC analysis.

Figure 3. Reaction requirements for 5-chlorouracil generation by reagent HOCl. Uracil was exposed to chloride-free HOCl (100 µM) in buffer A for 60 min at 37°C. Where indicated, chloride-free HOCl concentration (A), chloride concentration (B), pH (C), and reaction time (D) were varied. 5-Chlorouracil was quantified by HPLC analysis.

Figure 4. Electron capture negative ion GC/MS detection of the [M - PFB$^*$]$^-$ ions of pentafluorobenzylated 5-chlorouracil (A,B) and 5-bromouracil (C,D) in human inflammatory tissue. Selected ion chromatograms of endogenous 5-chlorouracil (A; m/z 325) and 5-bromouracil (C; m/z 371). Note co-elution of the ions of the analytes and internal standards. Full
scan mass spectra of the material eluting with the respective retention times of 5-chlorouracil (B; 4.75 min) and 5-bromouracil (D; 6.43 min) reveal the prominent M + 2 isotope patterns characteristic of brominated and chlorinated compounds, respectively.
Table 1. Production of 5-chlorouracil by peroxidases and other oxidation systems.

| Condition                                      | [5-chlorouracil], µM |
|------------------------------------------------|----------------------|
| Complete peroxidase system                     |                      |
| Myeloperoxidase                               | 15.2 ± 0.1           |
| Eosinophil peroxidase                         | < 0.1                |
| Lactoperoxidase                               | < 0.1                |
| Horseradish peroxidase                        | < 0.1                |
| Complete myeloperoxidase system minus          |                      |
| Myeloperoxidase                               | < 0.1                |
| H_{2}O_{2}                                    | < 0.1                |
| Cl⁻                                           | < 0.1                |
| Complete myeloperoxidase system plus          |                      |
| Azide (10 mM)                                 | < 0.1                |
| 3-Aminotriazole (10 mM)                       | < 0.1                |
| L-Methionine (6 mM)                           | < 0.1                |
| Catalase (200 nM)                             | < 0.1                |
| 4-Hydroxyphenylacetic acid (1 mM)             | 12.3 ± 0.2           |
| Other oxidation systems                       |                      |
| HOCl (50 µM)                                  | 52.4 ± 0.6           |
| FeSO_{4} (100 µ M)                             | < 0.1                |
| FeSO_{4} + H_{2}O_{2} (100 µM + 2 mM)          | < 0.1                |
| CuSO_{4} + H_{2}O_{2} (100 µM + 2 mM)          | < 0.1                |
| Glucose (100 mM)                              | < 0.1                |

The complete peroxidase system consisted of 1 mM uracil, 10 nM peroxidase, and 50 µM H_{2}O_{2} in buffer A. The reaction mixture was incubated at 37°C for 60 min. Where indicated, components were omitted or added. Reactions with other oxidants were carried out with 1 mM uracil for 24 h at 37 °C in buffer A that lacked DTPA. 5-Chlorouracil was quantified by HPLC. Values are the means and standard errors of three independent experiments with duplicate determinations per experiment.
Table 2. Antioxidant and nitrite effects on 5-chlorouracil generation by the myeloperoxidase-
H$_2$O$_2$-Cl$^-$ system.

| Antioxidant          | [5-Chlorouracil], µM (% inhibition) |
|----------------------|-------------------------------------|
| None                 | 16.0 ± 0.2 (0)                      |
| Ascorbate            | < 0.1 (99)                          |
| N-Acetyl cysteine    | < 0.1 (99)                          |
| Glutathione          | 0.3 ± 0.1 (98)                      |
| Taurine              | 2.8 ± 0.3 (82)                      |
| Sodium nitrite       | 5.8 ± 0.1 (64)                      |
| Trolox               | 9.6 ± 0.8 (40)                      |
| Phenol               | 11.3 ± 0.1 (29)                     |
| Mannitol             | 15.9 ± 0.2 (1)                      |

The complete myeloperoxidase system was as described in the legend to Fig. 2. Antioxidants and H$_2$O$_2$ were included at the same concentrations (50 µM). 5-Chlorouracil was quantified by HPLC analysis. Values are the means and standard errors of three independent experiments with duplicate determinations per experiment.
Table 3. Requirements for conversion of uracil into 5-chlorouracil by human neutrophils.

| Condition                                      | [5-Chlorouracil], nM |
|------------------------------------------------|----------------------|
| Complete system                                | 161 ± 10             |
| Complete system *minus*                        | < 10                 |
| Phorbol myristate acetate (200 nM)             | < 10                 |
| Cells                                          | < 10                 |
| Complete system *plus*                         | < 10                 |
| Azide (10 mM)                                  | < 10                 |
| L-Methionine (6 mM)                            | < 10                 |
| Catalase (10 µg/mL)                            | < 10                 |
| Superoxide dismutase (10 µg/mL)                | 186 ± 7              |

Neutrophils (1 x 10⁶/ml) suspended in buffer B supplemented with 1 mM uracil were activated with 200 nM phorbol myristate acetate and maintained in suspension with intermittent inversion at 37 °C for 60 min. The reaction was terminated by adding 6 mM methionine and centrifuging the cells at 400 x g for 10 min. 5-Chlorouracil was extracted from the supernatant, converted to its pentafluorobenzyl derivative, and analyzed by isotope dilution GC/MS with selected ion monitoring. Values are the means and ranges of two independent experiments, with duplicate determinations in each experiment.
A) Absorbance at 274 nm

- MPO
- MPO, +5-chlorouracil

Retention time (minutes)

B) Absorbance

Complete
5-Chlorouracil

nm

C) [M-CH₃]⁺

[M-Cl]⁺

[M-CH₃-HCl]⁺

m/z

Henderson, Fig1
Figure 4

(A) GC retention time (min) vs. ion current for m/z 331 (internal standard), m/z 325 (chlorouracil), and m/z 328 (artifact control).

(B) Mass spectrum showing [M-PFB\(^{+}\)]\(^{-}\) ions with m/z values of 324, 325, 326, 327, and 328.

(C) GC retention time (min) vs. ion current for m/z 377 (internal standard), m/z 371 (bromouracil), and m/z 374 (artifact control).

(D) Mass spectrum showing Br ions with m/z values of 368, 369, 370, 371, and 372.

Henderson, Fig 4
Phagocytes produce 5-chlorouracil and 5-bromouracil, two mutagenic products of myeloperoxidase, in human inflammatory tissue
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