5-Chlorouracil, a Marker of DNA Damage from Hypochlorous Acid during Inflammation

A GAS CHROMATOGRAPHY-MASS SPECTROMETRY ASSAY*

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Hypochlorous acid (HOCl), generated from H₂O₂ and Cl⁻ by myeloperoxidase in activated neutrophils, causes tissue damage during inflammation. We have developed a simple, sensitive (−0.2 fmol on column) and specific GC-MS assay for the detection of 5-chlorouracil (5-ClUra), a signature product of HOCl-mediated damage to nucleobases. In this assay, 5-ClUra is released from isolated DNA by a digestion with nuclelease P₁, alkaline phosphatase, and thymidine phosphorylase (TP), or from chlorinated nucleosides in biological fluids by TP. The freed 5-ClUra is derivatized with 3, 5-bis(trifluoromethyl)-benzyl bromide, which is detected by negative chemical ionization mass spectrometry. The assay can be used to simultaneously detect other halogenated uracils including bromouracil. Using this assay, we showed that 5-ClUra is generated by the reaction of low micromolar HOCl with (deoxy)cytidine, (deoxy)uridine, and DNA. In cell cultures, an increase of 5-ClUra was detected in DNA when cells were treated with sublethal doses of HOCl and allowed to proliferate. The elevation of 5-ClUra was markedly accentuated when physiologically relevant concentrations of (deoxy)uridine, (deoxy)cytidine, uracil, or cytosine were present in the medium during HOCl treatment. In the carrageenan-induced inflammation model in rats, chlorinated nucleosides was significantly increased, compared with controls, in the exudate fluid isolated from the inflammation site. Our study provides the direct evidence that chlorinated nucleosides are found in the inflammation site and can be incorporated in DNA during cell/tissue proliferation. These findings may be relevant to the carcinogenesis associated with chronic inflammation.

Chronic inflammation contributes to the development of cancer (1, 2). Oxidative modification of DNA bases may cause mutation and therefore could contribute to carcinogenesis. Hypochlorous acid (HOCl), generated from H₂O₂ and Cl⁻ by myeloperoxidase (MPO)† in activated neutrophils, plays a key role in the host defense against bacteria and other invaders (3). Consistent with this notion, the amount of intraphagosomal production of HOCl during phagocytosis is estimated to be sufficient to exert potent bactericidal activity (4), and MPO knock-out mice are susceptible to infection (5). However, despite its protective role against infection, excessive HOCl released during inflammation can contribute to tissue damage. Several biomarkers specific for HOCl-caused damage have been identified. Chlorination of cholesterol gives rise to cholesterol chlorohydrin (6). 3-Chlorotyrosine, a protein oxidation marker from HOCl or molecular chorine (7, 8), was found in inflammatory tissues and the lesions of inflammation-associated diseases, such as atherosclerosis (9).

In addition to proteins and lipids, HOCl also oxidizes nucleobases, which are electron-rich. It has long been recognized that bleach, composed mainly of sodium hypochlorite, oxidizes uracil to 5-chlorouracil (5-ClUra) (10). Whitman et al. (11) recently demonstrated that 5-ClUra can be produced by the reaction of HOCl with DNA, although relatively high concentrations of HOCl were used in the study. Henderson et al. (12) showed that under relatively acidic conditions (pH 4.5–6), HOCl, or MPO-H₂O₂-Cl⁻ oxidizes cytosine mainly to 5-chlorocytosine. 5-Chlorocytosine appears to be relatively unstable and converts to 5-ClUra by spontaneous (13) or enzymatic deamination (14). Therefore, 5-ClUra seems to be a specific marker of HOCl-caused damage to nucleobases in DNA.

Although these in vitro studies suggest that excessive generation of HOCl may lead to an increase in 5-ClUra during inflammation, due to the lack of a suitable assay, no evidence shows that this adduct is present in DNA of living cells after an insult of sublethal doses of HOCl. Herein we describe a simple, sensitive, and reliable GC-MS assay for the measurement of 5-ClUra. Using this assay, we found that 5-ClUra can be detected in the DNA isolated from cells that were treated with sublethal doses of HOCl, or in the inflammation fluid in a rat model.

EXPERIMENTAL PROCEDURES

Materials—Nuclease P₁ and alkaline phosphatase (AP) were purchased from Roche Applied Science. Thymidine phosphorylase (TP) (Sigma) was purified using a 10 kDa molecular cut-off filter (Millipore, Bedford, MA), followed by one-time washing with water and three times with 0.2 m KH₂PO₄ (pH 7.2), respectively. Triethylamine, acetonitrile, and 3,5-bis(trifluoromethyl)benzyl bromide (BTFMBzBr) were from Aldrich. RNase A and protease K were from Qiagen (Valencia, CA).

5-chlorouracil; 5-ClU; 5-chlorodeoxyuridine; dU, deoxyuridine; dC, deoxythymidine; AP, alkaline phosphatase; TP, thymidine phosphorylase; BTFMBzBr; 3,5-bis(trifluoromethyl)benzyl bromide; Thy, thymine; EI, electron impact; NCI, negative chemical ionization; PBS, phosphate-buffered saline; FBS, fetal bovine serum; GC-MS, gas chromatography-mass spectrometry.

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The abbreviations used are: MPO, myeloperoxidase; 5-ClUra, 5-Chlorouracil; 5-ClU, 5-chlorouracil; 5-CldU, 5-chlorodeoxyuridine; dU, deoxyuridine; dC, deoxythymidine; AP, alkaline phosphatase; TP, thymidine phosphorylase; BTFMBzBr, 3,5-bis(trifluoromethyl)benzyl bromide; Thy, thymine; EI, electron impact; NCI, negative chemical ionization; PBS, phosphate-buffered saline; FBS, fetal bovine serum; GC-MS, gas chromatography-mass spectrometry.
5-Chlorouracil

[15N2,13C]chlorouracil and [15N2,13C]uracil were purchased from Cambridge Isotope Laboratories, Inc (Andover, MA). Sodium hypochlorite, calf thymus DNA and other reagents were from Sigma.

**Synthesis of [15N2] 5-Chlorouracil**—[15N2, 13C]chlorouracil (~70 μmol) was added to an equimolar solution of sodium hypochlorite in phosphate-buffered saline (PBS) at pH 7.4 and incubated at 4 °C for 25 min. 5-[15N2, 13C]chlorouracil and non-reacted [15N2, 13C]uracil were separated on a Supelcosil LC-18, semipreparative, reverse phase HPLC column (5-μm particle size, 10 mm × 25 cm, Supelco, Bellafonte, PA), which was washed at 1 ml/min with 50 mM ammonium acetate at pH 6.0 with 5% methanol. 5-[15N2, 13C]chlorouracil was collected and dried by lyophilizer. Chemical identity of 5-[15N2, 13C]chlorouracil was obtained by matching HPLC retention time with authentic standard and confirmed by mass spectrometry. This procedure yielded high chemical purity (>98%) and isotopic purity (>99%) of 5-[15N2, 13C]chlorouracil.

**DNA Isolation**—DNA was isolated from cells and tissues using a Qiagen kit (69504, Valencia, CA) or phenol-chloroform extraction. In the latter method, nuclei were isolated from tissue homogenate by a published protocol (15), then digested with RNase A to remove RNAs, incubated with protease K to degrade proteins, and finally extracted with phenol and chloroform. DNA was then precipitated by ethanol in the presence of 0.3 M sodium acetate at pH 5.1. The amount of DNA was quantified by the picogreen assay (Molecular Probes, Eugene, OR).

**Enzymatic Hydrolysis of DNA**—20–150 μg of DNA, dissolved in 200 μl of 20 mM sodium acetate at pH 5.1, was digested to nucleotides by 3 μl of nuclease P1 under 65 °C for 10 min. The pH of the resulting solution was adjusted to ~8.0 using 40 μl of 0.1 M sodium borate at pH 8.7. Nucleosides were obtained on incubation with 2 μl of 4 units/μl alkaline phosphatase at 37 °C for 1 h. Thymine and 5-chlorouracil were released on incubation with 1 unit of purified thymidine phosphorylase (see “Experimental Procedures”) at 37 °C for 1 h, with the addition of 50 μl of 0.2 M potassium phosphate (pH 7.2).

**Extraction of Thymine and 5-ClUra**—After enzymatic digestion, 128 pg of 5-[15N2, 13C]chlorouracil and 95 ng of [15N2]thymine were added as internal standards. 1 ml of ethyl acetate was added into the resulting mixture, and free bases were extracted by vigorously shaking on a vortex mixer for 30 s. After a brief centrifugation, 0.85 ml of ethyl acetate was removed, and the ethyl acetate layer was collected, and the solvent was evaporated under N2.

**Derivatization**—After extraction and drying, the residue was suspended in 50 μl of acetonitrile, 5 μl of triethylamine, and 1 μl of 3,5-bis(trifluoromethyl)benzyl bromide (BTFMBzBr). The resulting mixture was incubated by vigorously shaking in a 124 shaker (New Brunswick Scientific Co, Inc., New Brunswick, NJ) at 37 °C for 25 min. After derivatization, 50 μl of water and 110 μl of isoctane were added. BTFMBz-5-chlorouracil and thymine derivative were extracted into isoctane by shaking vigorously on a vortex mixer for 30 s followed by a brief centrifugation. The top isoctane layer was collected and injected in a gas chromatograph-mass spectrometer.

**Gas Chromatography—Mass Spectrometry**—One microliter of sample in isoctane was injected onto a 5890 II gas chromatograph (GC) with a 7673 autosampler (Hewlett-Packard, Palo Alto, CA). GC was set up in the splitless mode with a purge activation time of 1 min and split vent flow of 50 ml/min. Ultra-high purity helium (Praxair, CA) was used as the carrier gas at a linear velocity of 55 cm/s. The injection port was maintained at 280 °C. An adequate separation of thymine and 5-chlorouracil and a good peak shape were obtained on a capillary column (30 m × 0.25 mm internal diameter × 0.25 mm film thickness [HP-5MS], Hewlett-Packard) by holding the oven at 100 °C for 1 min and then ramping to 220 °C at 20 °C/min and staying for 3 min, to 250 °C at 30 °C/min for 2 min, and to 350 °C at 40 °C/min and staying for 3 min. A 5998A mass spectrometer (Hewlett-Packard) was used for acquiring both electron impact (EI) and negative chemical ionization (NCI) mass spectra. EI data was obtained using an electron energy of 70 eV. NCI-MS was obtained using the following parameters: electron energy of 230 eV, ion source temperature of 250 °C, quadrupole temperature 300 °C, high energy electron 10 eV, and reagent gas (Matheson, Newark, CA) maintained at ion source temperature of 2.0–2.2 torr. The instrument was controlled using Hewlett-Packard Chemstation 1034C version C.01.05 software.

The maximum sensitivity was achieved by using the selective ion monitoring (SIM) in the NCI. In the SIM mode, m/z of 371 and 374 was used to detect and quantify BTFMBz-derivatized 5-ClUra and 5-[15N2, 13C]chlorouracil. In order to measure thymine, a 2500-time dilution was made before injection and m/z at 351 and 355 was used to quantify BTFMBz-thymine and BTFMBz-D3-thymine. The quantification was based on the ratio of the peak area of 5-ClUra and internal standard and the calibration curve. The level of 5-ClUra in DNA was expressed as the ratio of 5-ClUra and Thy (mol/mol) in DNA.

**Cell Culture Experiments**—Both human Hela cells and murine embryo C3H10T1/2 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were routinely kept in Dulbecco’s modified Eagle’s medium (DMEM) with 10% of fetal bovine serum (FBS), split, and seeded with ~60–70% confluence at 24 h before treated with 18, 30, and 50 μM of [15N2]chlorouracil in the Hank’s Balanced Saline solution (HBSS) at 37 °C for 10–30 min and then immediately harvested. In other experiments, after HOCI treatment, cells were replenished with DMEM with 5% FBS, or cells were treated with HOCI in DMEM with 5% FBS, and then further incubated for 24 h before harvesting.

**Detection of 5-ClUra in Mice Using the Air Pouch Inflammation Model in Rats**—The animal use protocol was approved by the animal care committee at Children’s Hospital Oakland Research Institute and was strictly followed. The details of the animal study was described elsewhere (16). Briefly, male Wistar rats (250–330 g) (Charles River, CA) were caged singly and routinely fed ad libitum with Purina Chow with free access to the tap water. An air pouch was created by a subcutaneous injection of 12 ml of sterile air into the intrascapular area of the rat’s back. Thirty hours later, 2 ml of 0.5% carrageenan (Sigma) or PBS was injected into the air pouch. Twenty hours after the induction of inflammation, the air pouch was lavaged with PBS without Ca2+/Mg2+ and the exudate fluid was collected. After a brief centrifugation, the supernatant was collected and DNA was extracted using a Qiagen kit. Samples were then processed, derivatized, and analyzed by GC-MS as described above.

**Statistics**—A non-paired Student’s t test was used in all conditions. A p value less than 0.05 was considered statistically significant.

**RESULTS**

**Development of GC-MS Assay for the Detection of 5-ClUra**—We previously developed a sensitive GC-MS method for the detection of uracil where uracil is derivatized with BTFMBzBr (17). Here we directly adapted this technique for the assay of 5-ClUra. Derivatization of 5-ClUra with BTFMBzBr yields a volatile, electron-capture derivative that is readily detected by gas chromatography coupled with EI- or NCI-MS. Fig. 1A illustrates the EI spectrum and structure of the derivatized compound, 5-ClUra-D3BTFMBz. A mass to charge ratio (m/z) at 598 corresponds to the molecular ion [M+] of this derivative. The major fragmented ions were found at 579 [M–F], 371 [M–BTFMBz], 328 [M–BTFMBz–COH], and 227 [BTFMBz]. In the NCI mass spectrum (Fig. 1B), the predominant peak was found at m/z 371, which resulted from a cleavage of one BTFMBz from the parental ion. It is noticeable that the two major isotopes of chloride atoms, i.e. 35 Cl and 37 Cl with natural isotopic abundance of 3:1, give rise to the characteristic peaks, as indicated, for instance, by the abundance ratio of 3:1 for the m/z 371 to 373 [M–BTFMBz] (Fig. 1). The EI and NCI fragmentations of the internal standard, [15N2, 13C]5ClUra-BTFMBz, were shifted up three mass units as a result of three heavier isotopes of chloride atoms, i.e. [53Cl37Cl Kolkata-Addik-R] and [53Cl35ClKolkata-Addik-R].

In order to effectively release 5ClUra from DNA, we have tried several methods. Although DNA can be completely hydrolyzed by formic acid at high temperature (11) (13), in order to achieve good sensitivity and reproducibility, multiple complicated purification and enrichment steps had to be subsequently performed (13). We also attempted to digest DNA using HF-pyridine at room temperature for 1 h. However, contaminates...
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from this reagent showed a significant signal at 371, which co-migrates with 5-ClUra on the chromatograph, and thus interfered with 5-ClUra detection. We then turned to the enzymatic hydrolysis. Uracil glycosylase, which removes uracil from DNA, failed to recognize 5-ClUra. TP, an enzyme documented to excise thymine and its analog, bromouracil, from the corresponding nucleosides (18), was then tried. We found that TP is able to effectively remove 5-ClUra from nucleosides (5-CldU or 5-ClUridine), but the commercially available TP contains significant amounts of contaminates which interfere with 5-ClUra detection. To eliminate contaminates, we purified the commercial TP by filtration (see “Experimental Procedures”). The purified enzyme proved to be satisfactory for the current use. Because 5-bromouracil can also be removed by TP from the corresponding brominated nucleoside (18), another halogenated adduct and inflammation-associated biomarker from eosinophil peroxidase-H₂O₂ -Br⁻ (19), and the BTTFMBz-derivated 5-bromouracil and 5-ClUra were separated well on the GC chromatography (Fig. 2), these two adducts can be readily measured simultaneously using the current assay.

Scheme I summarizes the assay for 5-ClUra from DNA or chlorinated nucleosides in biological fluids. For DNA, the procedure includes a digestion of isolated DNA to nucleotides by phosphodiesterase using AP, and a release of 5-ClUra and thymine by TP. In biological fluids, 5-ClUra is freed from chlorinated nucleosides in biological fluids. For DNA, the phosphate (with a final concentration of 50 mM). The complete excision of 5-ClUra from known amounts of 5-CldU or 5-Cl-Uridine (not shown). The presence of various amounts of DNA did not interfere with the release of 5-ClUra (Table I). The quantitative removal of thymine is evident from the fact that the amount of DNA measured by picogreen assay is equal to that calculated from the GC-MS assay where thymine is assumed to represent 29% of the total base (Fig. 3A).

The detection limit of the current assay is ~10–20 fg of 5-ClUra on column, with a signal to noise ratio of more than 5. The minimum reliable detected value from DNA is 0.1–0.3 ClUra/10⁶ Thy. A linear response curve was achieved from low picogram (Fig. 3B) to nanogram ranges (not shown).

5-ClUra Was Detected When Low Micromolar (1–10 μM) HOCl Reacts with Nucleosides and DNA—To test whether low micromolar HOCl leads to the production of detectable 5-ClUra, deoxyxystidine (dC), deoxyuridine (dU), and calf thymus DNA were incubated with 1–10 μM of HOCl in the presence of physiological concentrations of chloride ion. We observed a dose-dependent increase of 5-ClUra under the conditions examined (Fig. 4). At neutral pH, the reaction of HOCl with dU produced higher yields of 5-ClUra than with dC (Fig. 4A). This is likely due to the fact that dC may be primarily oxidized to the chloramine, in contrast to dU which can be directly converted to 5-ClUra (20). The yield from chlorination of dC is higher under slightly acidic conditions (pH 5), compared with pH 7, which is likely due to the formation of molecular chlorine from HOCl under acidic conditions, which then chlorinates dC to 5-chlorocytidine (12). Chen et al. (13) recently reported that 5-chlorocytidine is unstable and quickly deamidates to 5-chlorouridine. Besides dU and dC, uridine, and cytidine were oxidized by HOCl to generate 5-ClUra with a similar product yield (data not shown). A dose-dependent increase of 5-ClUra was detected from calf thymus DNA when treated with 1–10 μM of HOCl (Fig. 4B), presumably as a result of the chlorination of deoxycytidine.

Elevated Levels of 5-ClUra Were Detected in the DNA of Proliferating Cells that Were Treated with Sublethal Doses of HOCl and Allowed to Proliferate, but not after an Immediate Insult—To test whether 5-ClUra can be generated by the direct insult of HOCl in live cells, both human Hela cells and murine embryo C3H10T1/2 cells were treated with non-lethal doses of HOCl. Immediately after HOCl (0–40 μM) treatment, no significant increase in 5-ClUra was detected from isolated DNA (Fig. 5A). At doses lower than 40 μM, no apparent cell death was observed as assayed by the trypan blue exclusion (data not shown). Even at higher dose of HOCl, e.g. 100 μM for 10 min, where substantial amounts of cells became detached or stained in the trypan blue exclusion assay, no significant increase of 5-ClUra could be found in isolated DNA (data not shown). On the other hand, after cells were treated with HOCl for 20 min, replenished with complete medium with FBS and further incubated for 20–24 h, a mild but significant increase in 5-ClUra was detected in the isolated DNA (Fig. 5A). Similar results were also obtained with Hela cells, not shown). The elevation of 5-ClUra was markedly accentuated by the addition of 1 μM dU and dC during HOCl treatment (Fig. 5B). This intensified elevation of 5-ClUra in DNA was blocked up to ~98% with the addition of 0.5 mM of methionine, a potent scavenger of HOCl. The presence of complete medium plus 5% FBS and dU or dC during HOCl treatment resulted in a reduction of 5-ClUra (Fig. 5C) compared with HBSS as the medium. Besides dU and dC, the addition of low micromolar uracil, cytosine, uridine, and cytidine to the culture medium during HOCl treatment also led to significant elevations of 5ClUra in the DNA (Fig. 5D). These observations suggest that although HOCl may not penetrate to the nuclei to directly oxidize DNA, it could modify dC, dU, uracil, cytosine, uridine, and cytosine in the surrounding tissues to generate chlorinated adducts, which might be incorpo-
rated into the DNA during cell growth.

To test the incorporating efficiency of chlorinated adducts into the DNA, 10 pM to 10 μM of 5-CldU or 5-ClUra were added to the medium when cells were in their log growth phase. As low as 10 pM of 5-CldU was effectively incorporated into DNA with an overall efficiency of 30–40% (Table II). On the other hand, the direct incorporation yield for 5-ClUra was more than 10⁴–10⁵-fold lower than that of 5-CldU (Table II).

**Elevated Levels of Chlorinated Uracil Adducts Were Detected in the Exudate Fluid at the Inflammation Site in a Rat Inflammation Model**—To test whether 5-ClUra is elevated during inflammatory response in vivo, we measured this adduct in the exudate fluid and the DNA from tissues isolated at the inflammation site in the carrageenan-induced inflammation model in rats, which is believed to mimic joint diseases (16, 21). In this model, a single injection of carrageenan in the pre-created air pouch in the intrascapular area of rat backs led to localized inflammation as indicated by a marked increase in neutrophil infiltration, pro-inflammatory eicosanoids and oxidative damage (16, 21). A markedly enhanced neutrophil infiltration to the inflammation site has been previously reported (21) and was also confirmed by us (16). At the time when

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**Table I**

| DNA     | 5-CldU added | 5-ClUra measured | Efficiency |
|---------|--------------|------------------|------------|
| μg      | pg           | pg               | %          |
| 0       | 7.2          | 3.9              | 97         |
| 20      | 7.2          | 4.1              | 97*        |
| 50      | 7.2          | 4.1              | 90*        |
| 100     | 7.2          | 4.4              | 90*        |

**SCHEME 1**

**Fig. 2.** Gas chromatograph of BTFMBz-derivatized Thy, 5-ClUra, and 5-bromouracil (BrUra) and the major ions of chlorinated and brominated uracil on the NCI (insets).
samples were collected, neutrophils accounted for >90% of the total cells in the exudate. Using the current GC-MS assay, we detected a significant increase in 5-CIUra in the isolated exudate fluid in carrageenan-treated rats, compared with PBS-injected controls (Fig. 6). It is important to note that the significant increase in 5-CIUra was observed only after the fluid was treated with TP, which releases 5-CIUra from 5-ClU or 5-Cl-uridine, suggesting that the predominate chlorinated adducts may be chlorinated nucleosides, but not 5-CIUra. We did not observe a statistically significant elevation of chlorination in the DNA that was isolated from the inflammation site tissues (data not shown).

DISCUSSION

We have developed a simple, specific, and sensitive assay for the detection of 5-CIUra. The assay employs thymidine phosphorylase-catalyzed de-pyrimidination to achieve a complete release of derivatizable 5-CIUra and thymine from DNA under physiological condition. The specificity of the enzymatic cleavage and the subsequent separation of freed bases from the nucleosides by a one-step ethyl acetate extraction (Scheme 1), result in a simple and clean product profile, which is critical for obtaining an efficient and reproducible derivatizing outcome and high sensitivity. Our assay is thus simpler and yet highly sensitive, compared with a recently published GC-MS assay (13), which uses acid hydrolysis to completely digest DNA, and involves multiple clean-up and enriching steps in order to achieve a similar sensitivity. Our assay is also capable of measuring chlorinated nucleosides in biological fluids (Fig. 6), which are difficult to directly analyze by acid hydrolysis. In addition, the simultaneous removal of both 5-CIUra and thymine by TP makes it convenient to use thymine to estimate the amount of DNA. The assay can simultaneously measure chlorinated and brominated uracil, while the specificity and sensitivity for 5-CIUra is particularly valuable because an equally sensitive and specific ELISA (enzyme-linked immunosorbent assay) is not likely to be available due to the poor antigenicity of the chloride atom. In contrast, ELISA assays are commonly available for detecting 5-bromouracil, due to the strong antigenicity of bromide atom. Although TP indistinguishably releases 5-CIUra from 5-ClU or 5-Cl-Uridine, in the current study, RNA was completely removed by the digestion with RNase A under DNA isolation (data not shown). On the other hand, the method can be adapted to measure the chlorinated and brominated nucleobases in RNA, the feasibility of which is currently under investigation.

Previous studies together with our current investigation indicate that 5-CIUra appears to be a sensitive, specific and stable marker of HOCl-caused damage. It has been shown that the reaction of HOCl with nucleobases primarily leads to extensive damage to pyrimidines such as cytosine, uracil and thymine, but minor damage to purines (11, 12, 22). HOCl oxidizes uracil to 5-CIUra (20), and primarily oxidizes cytosine to chloroamines under neutral pH or 5-chlorocytosine at acidic pH (12). Chloramine-cytosine slowly gives rise to other base lesions, including 5-CIUra (23–25). 5-Chlorocytosine is also
Qiagen kit. containing 5-CldU or 5-ClUra at indicated concentrations and incubated in T-75 flask. Cells were then re-fed with 20 ml of fresh medium for 20 h. DNA was then isolated from the harvested cells using Qiagen kit.

| Added bases | 5-CIUr/Thy | 5-CIUr calculated | Incorporation |
|-------------|------------|-------------------|--------------|
| 5-CldU (μM) | mol/10^6 mol | pg in 20 μg of DNA | %            |
| 0           | 0.25       | 0.61              | –            |
| 10          | 5.45       | 12.2              | 42           |
| 100         | 43.7       | 98.2              | 34           |
| 1000        | 378        | 850.4             | 29           |
| 5-CIUr (μM) | 1          | 2.17              | 2.2 × 10^-6  |
|             | 10         | 17.4              | 1.8 × 10^-4  |

TABLE II
Picomolar concentrations of 5-CldU, but not 5-CIUr, were efficiently incorporated into C3H10T1/2 cells

Murine embryo C57BL/10T1/2 cells were grown to reach 50–60% confluency in T-75 flask. Cells were then re-fed with 20 ml of fresh medium containing 5-CldU or 5-CIUr at indicated concentrations and incubated for 20 h. DNA was then isolated from the harvested cells using Qiagen kit.

...relatively unstable and spontaneously deaminates (13) or during acid hydrolysis to generate 5-CIUr (11, 13). In biological systems, cytidine deaminase readily converts 5-chloro-dC to 5-chloro-dU and the apparent half-life of 5-chloro-dC is about 10 min in vitro (14). On the other hand, 5-CIUr seems to be quite stable because it is resistant to further modification by acid hydrolysis or strong oxidants including peroxynitrite (26). Therefore, 5-CIUr appears to be a stable and specific product of HOCl-mediated modification of cytosine and uracil. In the current study, we find that low micromolar HOCl oxidizes (deoxy)uridine or (deoxy)cytidine and DNA to generate detectable 5-CIUr at both pH 7 and pH 5. Under slightly acidic conditions (pH 5), HOCl-mediated chlorination of dC yields similar amounts of 5-CIUr compared with that of dU (Fig. 4), suggesting that both (deoxy)uridine and (deoxy)cytidine contribute significantly to the generation of 5-CIUr. Because our assay does not involve acidic hydrolysis, 5-CIUr may be directly derived from chlorinated cytosine by way of chloramine conversion and/or deamination of 5-chlorocytosine. Although high micro- to millimolar HOCl has been estimated at the site of inflammation or inside phagosomes (4), the scavenging by antioxidants and other biomolecules makes low micromolar HOCl more likely to be relevant to the levels that could react with nucleobases in complex biological environments. Thus, in addition to being stable and specific, 5-CIUr is a sensitive marker for HOCl-mediated damage to nucleotides, nucleosides, free nucleobases, DNA, and RNA.

Although HOCl chlorinates nucleobases in DNA in vitro, considering its high reactivity and cytotoxicity (27), the question regarding the physiological importance of HOCl-mediated DNA damage is whether the chlorinated adduct can be found in the nuclear DNA of live cells after being treated with non-lethal doses of HOCl. In the earlier stage of the study, we did not observe a significant increase of chlorinated adduct in the DNA immediately after cells were treated with HOCl, possibly because HOCl is scavenged by defense biomolecules such as glutathione (28) before reaching nuclei and directly oxidizing DNA. On the other hand, we observed an elevation of 5-CIUr after a prolonged incubation subsequent to the HOCl treatment, suggesting that chlorinated adducts, which may be derived from HOCl-oxidized cytosolic nucleotide/nucleoside pools, are incorporated into the DNA during cell proliferation. This mechanism is confirmed by the observation that the addition of physiologically relevant concentrations of nucleosides and nucleobases to the media during HOCl treatment causes a markedly intensified increase of 5-CIUr in the isolated DNA (Fig. 5). The presence of methionine, a HOCl scavenger, almost completely abolishes this increase, although a significant elevation of 5-CIUr is found even in the presence of complete growth medium during HOCl treatment. We further compared the relative efficiency for 5-CldU and 5-CIUr to be incorporated into DNA. In this regard, incorporation of 5-CldU is much more efficient than that of 5-CIUr, suggesting that at similar concentrations, chlorinated deoxynucleosides, but not nucleobases, are likely to be the major precursors to be incorporated. Our cell culture experiments can mimic the situation at the site of inflammation. Under physiological conditions, the plasma concentrations of uracil, cytosine, uridine, and cytidine are in the low micromolar ranges (29). During inflammation, nucleosides and nucleobases in the plasma exude to the inflammatory site and become the targets of HOCl, which is generated by the MPO-H2O2-Cl− system of infiltrated neutrophils. The chlorinated nucleosides or bases may then be incorporated into proliferative tissues.

We have detected a significant increase of 5-CIUr in the exudate fluid isolated from the inflammation site in which infiltrated neutrophils are particularly abundant (Fig. 6). Interestingly, in the current study, the enhanced 5-CIUr at the site of inflammation is likely derived from chlorinated nucleosides instead of free bases because a significant increase of 5-CIUr is detected only after samples were de-pyrimidinated by TP. Despite an increase of chlorinated adducts in the inflammatory fluid, we did not detect significant enhancement of 5-CIUr in the DNA isolated from the inflammatory tissues. This may be explained by the possibility that the tissues, when isolated, are not undergoing proliferation. In the future, tissues from a chronic inflammation site or undergoing proliferation subsequent to the inflammation should be tested for the chlorinated products in DNA.

It is conceivable that during chronic inflammation, chlorinated nucleosides may be frequently generated and incorporated into the DNA of proliferating tissues. This may lead to the accumulation of chlorinated bases in the DNA especially because halogenated uracils, the thymine analog, are not efficiently repaired (30). The accumulation of 5-CIUr in DNA has been shown to induce sister chromatid exchange, and mutations (30). Therefore, chronic inflammation-mediated accumulation of modified bases may be relevant to the development of cancer.

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Addendum—After this manuscript was submitted, Henderson et al. published an article (Henderson, J. P., Byun, J., Takeshita, J., and Heinecke, J. W. (2003) J. Biol. Chem. 278, 23522–23528) showing that 5-chlorouracil can be generated from uracil by MPO-H2O2-Cl− or activated neutrophils through a mechanism involved HOCl as the interme-
They also found enhanced levels of 5-ClUra and 5-bromouracil in human inflammatory tissues. Their study complements our current observations that physiologically relevant concentrations of HOCl oxidize uracil, cytosine, and their nucleosides to generate chlorinated adducts, which are found in the inflammation sites in a rat inflammation model and can be incorporated into nuclear DNA during cell/tissue proliferation.

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