

## Genotoxic effects of neutrophils and hypochlorous acid

Nejla Güngör<sup>1</sup>, Ad M. Knaapen<sup>1,4</sup>, Armelle Munnia<sup>2</sup>,  
Marco Peluso<sup>2</sup>, Guido R. Haenen<sup>3</sup>, Roland K. Chiu<sup>1</sup>,  
Roger W. L. Godschalk<sup>1</sup> and Frederik J. van Schooten<sup>1\*</sup>

<sup>1</sup>Department of Health Risk Analysis and Toxicology, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Maastricht University, PO Box 616, 6200 MD, Maastricht, The Netherlands, <sup>2</sup>Cancer Risk Factor Branch, Molecular Biology Laboratory, ISPO-Cancer Prevention and Research Institute Florence, Via Cosimo il Vecchio 2, 50139 Florence, Italy and <sup>3</sup>Department of Pharmacology and Toxicology, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Maastricht University, PO Box 616, 6200 MD, Maastricht, The Netherlands

**Chronic inflammation has been recognized as a contributing factor in the pathogenesis of lung cancer. In this process, reactive oxygen species released by neutrophils may play an important role. The aim of the present study was to investigate the capacity of the major neutrophilic oxidant hypochlorous acid (HOCl), which is formed by myeloperoxidase (MPO), to induce DNA damage and mutagenicity in lung cells. HOCl was mutagenic in lung epithelial A549 cells *in vitro*, showing at physiological concentrations a significant induction of mutations in the *HPRT* gene. We studied three major types of DNA lesions that could be relevant for this HOCl-induced mutagenicity. Single strand DNA breakage and 8-oxo-7,8-dihydro-2'-deoxyguanosine were not found to be increased following HOCl treatment. On the other hand, HOCl caused a significant increase in the formation of 3-(2-deoxy- $\beta$ -D-erythro-pentofuranosyl)pyrimido[1,2- $\alpha$ ]purin-10(3H)-one (M<sub>1</sub>dG), which can be formed by either malondialdehyde (MDA) or base propenals. We observed an increased MDA formation upon exposure of A549 cells to HOCl, but a role of base propenals cannot be excluded. In line with this, we observed 4-fold increased M<sub>1</sub>dG adduct levels in mice that were intratracheally instilled with lipopolysaccharide to induce a pulmonary inflammation with neutrophil influx. Depletion of circulating neutrophils significantly reduced pulmonary MPO activity as well as M<sub>1</sub>dG adducts levels, thereby providing a causal link between neutrophils/HOCl and pulmonary genotoxicity *in vivo*. Taken together, these data indicate that MPO catalysed formation of HOCl during lung inflammation should be considered as a significant source of neutrophil-induced genotoxicity.**

### Introduction

A significant fraction of the global cancer burden is attributable to chronic inflammation (1). For instance, pulmonary inflammation linked to smoking is widely recognized as a risk factor for lung cancer development. For the respiratory tract,

however, the mechanism of carcinogenesis associated with chronic inflammation has not yet been fully elucidated. Alveolar and bronchial epithelial cells are exposed to a variety of reactive oxygen species (ROS) from both exogenous (e.g. cigarette smoke) and endogenous sources, such as the phagocytic respiratory burst. With regard to tumour formation, it has been postulated that ROS, produced by the prolonged activation of inflammatory polymorphonuclear neutrophils, play a role in lung tumour formation (2). But also in later stages of the carcinogenic process, during tumour progression, neutrophil infiltration and associated release of ROS in tumours provide a growth advantage (3). Upon activation, neutrophils induce various oxidant-generating enzymes, such as NADPH oxidase, superoxide dismutase and myeloperoxidase (MPO). As a result, high concentrations of a variety of ROS are generated, including superoxide (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and the MPO-catalysed production of hypochlorous acid (HOCl), which are produced to attack invading infectious agents (4). However, excess amounts of ROS generated in inflamed tissues can also damage DNA in target cells, leading to increased mutation load and potentially carcinogenesis (5–8).

In rats, chronic exposure to particles, inducing a massive recruitment of neutrophils, is associated with tumour formation, and neutrophil-induced genetic alterations and mutagenic effects in lung epithelial cells are thought to be key factors in this process (9,10). *In vitro* coincubation of rat lung epithelial cells with bronchoalveolar lavage (BAL) cells, isolated from particle-treated rats, increased mutation frequency in the hypoxanthine guanine phosphoribosyl transferase (*HPRT*) gene (11). Previously reported studies, using similar *in vitro* coincubation models of target cells with activated neutrophils, demonstrated that neutrophils are able to induce sister chromatid exchanges (5), strand breaks (12) and mutations in neighbouring target cells (reviewed in ref. 8), suggesting a role of neutrophil-derived ROS. In a previous study, we demonstrated that activated neutrophils cause induction of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) in alveolar cells *in vitro*, suggesting involvement of hydroxyl radicals (<sup>•</sup>OH) (13). Altogether, these studies suggested that H<sub>2</sub>O<sub>2</sub> generated by neutrophils is one of the essential mediators of the genotoxic effects, as it serves as latent form of hydroxyl radicals. H<sub>2</sub>O<sub>2</sub> is very stable and can cross cellular membranes and is at the same time able to penetrate the nucleus where <sup>•</sup>OH can be generated in the vicinity of the DNA molecule via the Fenton reaction with localized transition metals (14). However, under physiological conditions, up to 70% of H<sub>2</sub>O<sub>2</sub> is consumed by the neutrophil-derived enzyme MPO to generate the strong oxidant HOCl (15). Interestingly, MPO has been implicated in the pathogenesis of inflammation-related carcinogenesis, with reference to the -463G → A polymorphism in the promoter region of the *MPO* gene, which is associated with a reduced

\*To whom correspondence should be addressed. Tel: +31 43 3881100, Fax: +31 43 3884146, Email: f.vanschooten@grat.unimaas.nl

<sup>4</sup>Present address: Department of Toxicology and Drug Disposition, Schering-Plough, PO Box 20, 5340 BH Oss, The Netherlands

risk of cancer in the lung (16,17). Nevertheless, there are only limited data available on a potential role of HOCl in the pathophysiology of inflammation-related lung cancer development.

The aim of the present study was to assess the DNA damaging and mutagenic effects of neutrophil-derived HOCl. Firstly, we assessed three different types of HOCl-induced DNA damage [DNA single-strand breakage (ssDNA), 8-oxo-dG and 3-(2-deoxy- $\beta$ -D-erythro-pentofuranosyl)pyrimido[1,2- $\alpha$ ]purin-10(3H)-one (M<sub>1</sub>dG), as well as *HPRT* mutations in human alveolar epithelial lung cells (A549)]. Secondly, based on initial *in vitro* findings revealing M<sub>1</sub>dG adducts as the most significant HOCl-induced DNA damaging effect *in vitro*, we assessed this promutagenic DNA adduct formation in the mouse lung following acute lipopolysaccharide (LPS) instillation. The causal role of neutrophils in this *in vivo* genotoxic effect was addressed by depleting the animals of circulating neutrophils.

## Materials and methods

### Cell treatment with HOCl

Human epithelial lung adenoma carcinoma cell line (A549) (American Tissue Culture Collection) was cultured in Dulbecco's modified Eagle's medium (Sigma, Zwijndrecht, The Netherlands) supplemented with 10% heat inactivated foetal calf serum (Gibco Invitrogen, Breda, The Netherlands) and 1% penicillin-streptomycin (Sigma) under humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. Cell passages between 25 and 40 were used for experiments described in this study.

The concentration of HOCl stock solution (Sigma) was measured spectrophotometrically, immediately before use, at 293 nm, using the extinction coefficient of hypochlorite  $\epsilon_{293} = 349.2/\text{M}\cdot\text{cm}$  at pH 10–12. For comet assay and DNA isolation, nearly confluent monolayers of A549 cells grown in 75 cm<sup>2</sup> culture flasks were used. Medium was discarded and cells were exposed to increasing concentrations of HOCl (0–100  $\mu\text{M}$ ) suspended in Hanks' balanced salt solution (HBSS). After 15 min, cells were harvested by trypsinization. Acute cytotoxicity of HOCl in A549 cells was tested using trypan blue dye exclusion. For the lipid peroxidation assay, A549 cells were cultivated in 100 mm dishes and treated at near confluency as described above. After the 15 min incubation period, medium was removed and the dishes were kept on ice. Cell monolayers were washed with ice-cold phosphate-buffered saline (PBS) and scraped from the dishes with 500  $\mu\text{l}$  of fresh PBS per dish. Cells were sonicated briefly (15 sec) and the lysate was stored at  $-20^\circ\text{C}$  and used for malondialdehyde (MDA) measurement by thiobarbituric acid reactive substances (TBARS) formation.

H<sub>2</sub>O<sub>2</sub> (250  $\mu\text{M}$ ) was used as a positive control for ssDNA, 8-oxo-dG and M<sub>1</sub>dG. Positive control for MDA measurement was Fe<sup>2+</sup> (10  $\mu\text{M}$ ). Benzo[*a*]pyrene-diol-epoxide (BPDE, 0.2 and 1  $\mu\text{M}$ ) was used as positive control for *HPRT* mutation analysis.

### Single cell gel electrophoresis

DNA single-strand breakage in A549 cells was determined by alkaline comet assay (18), implemented according to recent guidelines (19). After the incubation, cells were washed once, harvested and resuspended in HBSS at a cell concentration of  $2 \times 10^6$  cells per ml. The cell suspension was mixed with low-melting point (LMP) agarose (Sigma), reaching a final concentration of 0.65% LMP agarose and positioned on 1.5% agarose-coated slides. Further procedures were as described earlier (20). Comets were stained with ethidium bromide (10  $\mu\text{g}/\text{ml}$ ) for fluorescence microscopy: per blindly scored slide, a total of 50 cells were scored using the software program Comet Assay III (Perceptive Instruments Ltd, Haverhill, UK). DNA strand breakage data were expressed as Tail Moment, which is defined as the product of DNA content in the tail and the mean distance of migration in the tail.

### DNA isolation

DNA was isolated using standard phenol extraction (21). The DNA extraction procedure was optimized to minimize artificial induction of 8-oxo-dG by using radical-free phenol, minimizing exposure to oxygen and by adding 1 mM deferoxamine mesylate and 20 mM 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO), according to the European Standards Committee on Oxidative DNA Damage (22). DNA concentrations were quantified by NanoDrop (Isogen-Lifescience, De Meern, The Netherlands) and samples were frozen at  $-20^\circ\text{C}$  until further analysis.

### Quantification of 8-oxo-dG

High-pressure liquid chromatography with electrochemical detection (HPLC-ECD) of 8-oxo-dG was based on a method as described earlier (23). Briefly, after extraction, DNA was digested into deoxyribonucleosides by treatment with nuclease P1 (1 U/ $\mu\text{l}$ ) and alkaline phosphatase (0.014 U/ $\mu\text{l}$ ). The digest was then analysed by HPLC-ECD, using Supelcosil™ LC-18S column (250  $\times$  4.6 mm) (Supelco Park, Bellefonte, PA, USA) and DECADE electrochemical detector (Antec, Leiden, The Netherlands). The mobile phase consisted of 10% aqueous methanol containing 94 mM KH<sub>2</sub>PO<sub>4</sub>, 13 mM K<sub>2</sub>HPO<sub>4</sub>, 26 mM KCl and 0.5 mM ethylenediaminetetraacetic acid (EDTA). Elution was performed at a flow rate of 1 ml/min. 8-Oxo-dG was detected at a potential of 400 mV and dG was simultaneously monitored by UV absorption at 260 nm. Results were expressed as the ratio of the determined 8-oxo-dG to dG.

### Quantification of M<sub>1</sub>dG

The <sup>32</sup>P-postlabelling technique was used to analyse the levels of M<sub>1</sub>dG adducts, as previously reported (24). Briefly, a digestion method based on the DNA hydrolysis by micrococcal nuclease (Sigma) and a mixture of two spleen phosphodiesterases (Sigma) was applied. Hydrolysed samples, evaporated to dryness, were treated with nuclease P1 (Sigma). The modified nuclease P1-resistant nucleotides, evaporated to dryness, were incubated with carrier-free [ $\gamma$ -<sup>32</sup>P]-adenosine triphosphate (Amersham, Buckinghamshire, UK) and T4-poly nucleotide kinase (Epicentre Technologies, Madison, WI, USA) to generate <sup>32</sup>P-labelled adducts. M<sub>1</sub>dG adduct analysis was carried out by polyethyleneimine-cellulose thin-layer chromatography according to published conditions (25). Detection and quantification of M<sub>1</sub>dG adducts and total nucleotides were obtained by phosphor imaging technology (Typhoon 9210, Amersham) and ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). After background subtraction, the levels of DNA adducts were expressed as relative adduct labelling (RAL = adducted nucleotides/total nucleotides). Standard MDA-modified DNA (26) and -unmodified DNA were routinely processed in the analysis as controls.

### Lipid peroxidation assay

The TBARS were measured by means of HPLC according to Lepage *et al.* (27). Briefly, 100  $\mu\text{l}$  of cell lysate or MDA standard was mixed with 900  $\mu\text{l}$  reagent composed of 10 parts of reagent A [0.012 M 2-thiobarbituric acid (TBA), 0.32 M H<sub>3</sub>PO<sub>4</sub> and 0.01% EDTA] and one part of reagent B (butylated hydroxytoluene in ethanol, 1.5 mg/ml). Standards were prepared using 0–10  $\mu\text{M}$  MDA solutions in PBS, which were derivatized in the same analytical measurements as the samples. The tubes were covered with marbles and heated for 1 h at 99°C. After cooling, the product was extracted in 500  $\mu\text{l}$  butanol by vigorous shaking and the tubes were centrifuged for 5 min at 5000 *g*. In total, 30  $\mu\text{l}$  of the extract was injected on to a Nucleosil™ C18 column (150  $\times$  3.2 mm) (Supelco Park, Bellefonte, PA, USA) and eluted with 65% water and 35% methanol + 0.05% trifluoroacetic acid. Fluorescence was recorded at  $\lambda_{\text{ex}}$  553 nm. The peak of TBA-MDA product was integrated and the concentration of MDA was corrected for protein content of the lysates. The results were expressed as a percentage of TBARS formation, taking the control sample as 100%.

### Protein determination

Protein concentrations were determined spectrophotometrically using the DC-protein assay Kit (BIO-RAD, Veenendaal, The Netherlands) as per manufacturer's protocol.

### Clonogenic survival assay

The sensitivity of A549 cells to HOCl was assayed by clonogenic survival. Briefly, cells were seeded at a density of  $4 \times 10^5$  cells per 60 mm dish and grown for 24 h before exposure to HOCl (0–100  $\mu\text{M}$ ) or BPDE (1  $\mu\text{M}$ ). Cells were treated for, respectively, 15 and 30 min at 37°C with the indicated dosages, after which they were harvested and seeded in triplicate for each concentration. After  $\sim$ 14 days of growth, the medium was removed and colonies were fixed and stained with 2% bromophenolblue in 70% ethanol. Any groupings of cells containing 50 or more cells were counted as a colony. For each dose, survival was calculated from the relative colony-forming ability of the HOCl-exposed cells compared to control.

### HPRT mutation analysis

For the determination of *HPRT* mutant frequencies, A549 cells were seeded at  $1 \times 10^6$  cells per 100 mm dish. The following day, cells were treated with the different doses of HOCl (15 min at 37°C) or BPDE (30 min at 37°C) and were subcultured for 1 week to allow phenotypic expression of the acquired mutations. Cells were then seeded in selective medium containing 6-thioguanine (6-TG) and incubated until colonies were formed ( $\sim$ 3 weeks). 6-TG resistant colonies

consisting of cells carrying *HPRT* mutations were fixed, stained and counted. All experiments were normalized for plating efficiency. Finally, *HPRT* mutant frequency was defined after correcting for clonogenic survival.

#### LPS induced acute lung inflammation mouse model

Male wild-type (Wt) C57Bl6 mice (~12-week-old) were obtained from Charles River Breeding Laboratories (Heidelberg, Germany). Mice were housed individually in standard laboratory cages and received food and water *ad libitum* throughout the experiments. The studies were carried out in accordance with an approved protocol by the Institutional Animal Care Committee of Maastricht University.

Mice were treated with LPS (*Escherichia coli*, serotype O55:B5, Sigma) by intratracheal instillation to induce an acute pulmonary inflammation. The dose of LPS was 20 µg per instillation per mouse. Intratracheal instillation was performed by a non-surgical technique under anaesthesia as previously described (28). Sham mice were instilled with sterile 0.9% NaCl.

Since a previous study (29) demonstrated that local LPS challenge in Wt C57Bl6 mice results in a time-dependent neutrophil accumulation peaking at day 3, mice ( $n = 10$ ) were sacrificed 3 days post-exposure by 115 mg/kg sodium pentobarbital (Ceva Sante Animale, Maassluis, The Netherlands) and BAL (3× with 1 ml sterile 0.9% NaCl) was performed to remove as much as possible inflammatory cells from the airways. After centrifugation, the cell-free bronchoalveolar lavage fluid (BALF) was stored at  $-80^{\circ}\text{C}$  for MPO activity measurement. Lavaged lungs were snap frozen in liquid nitrogen and pulverized using mortar and pestle. The pulverized lung tissue was stored at  $-80^{\circ}\text{C}$  until DNA isolation.

To unravel the direct role of neutrophils in pulmonary genotoxicity/mutagenicity, depletion of circulating neutrophils in mice ( $n = 5$ ) was achieved by intraperitoneal injection of 0.5 mg of the monoclonal rat anti-mouse neutrophil antibody NIMP-R14 (Hbt, Maastricht, The Netherlands), 24 h before intratracheal LPS instillation. NIMP-R14 has been shown to selectively deplete mouse neutrophils *in vivo* for up to 6 days (30,31). In our study, a lack of MPO activity in the lung was used as a verification of this neutrophil absence. IgG (eBioscience, San Diego, CA, USA) was used as control monoclonal antibody in the sham group. Again, 3 days after instillation, mice were sacrificed, and lungs were lavaged and isolated as described above.

#### MPO activity measurement

Only activated neutrophils, which undergo an oxidative burst with associated release of MPO and subsequent formation of HOCl, can induce genotoxic effects in pulmonary epithelial tissue. Therefore, extracellular MPO activity was measured in cell-free BALF as described by Klebanoff *et al.* (32), which is indicative for the presence of (activated) neutrophils in the lung (33).

#### Statistical analysis

Data are expressed as mean  $\pm$  standard error of the mean (SEM) of duplicate determinations of at least two independent incubations concerning the *in vitro* experiments or of at least five *in vivo* samples per group. Analysis of variance with *post hoc* testing (Dunnnett's correction for multiple testing, two sided) was applied to test differences between treatments and control. Statistical analysis was performed using SPSS v.15.0. In each case, a difference was considered statistically significant at  $P < 0.05$ .

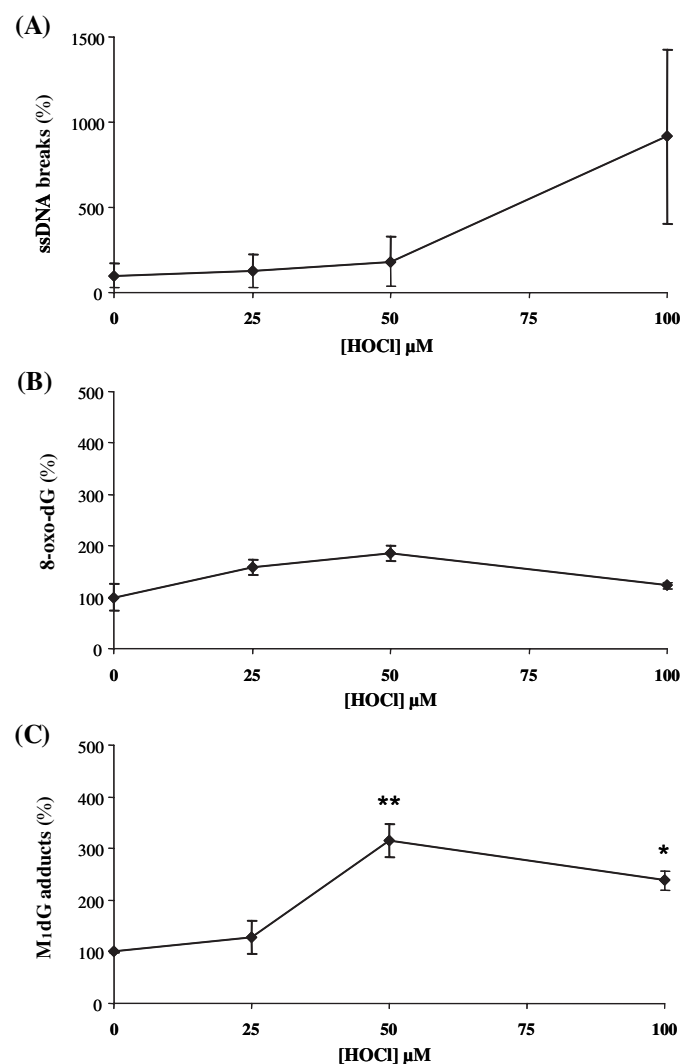
## Results and discussion

### Genotoxic effects of HOCl: use of an *in vitro* model

In previous coincubation models of neutrophils and lung epithelial cells, we showed that activated neutrophils induce the formation of 8-oxo-dG and ssDNA breaks in epithelial cells, which could be mimicked by treatment of the cells with  $\text{H}_2\text{O}_2$  (8,20). In the present study, we investigated the ability of the major oxidant derived from activated neutrophils, namely HOCl, to induce ssDNA breaks, 8-oxo-dG and  $\text{M}_1\text{dG}$  in A549 cells. When A549 cells were exposed to increasing concentrations of HOCl, no acute cytotoxic effects were observed at physiological concentrations. The viability of the epithelial cells was  $>90\%$  after treatment with up to 200 µM of HOCl during 15 min. These doses can be produced *in vivo* at sites of inflammation and also reflect the amount of HOCl produced in *in vitro* co-cultures (1:1 ratio with activated neutrophils yields  $\sim 150$  µM HOCl) (34).

At physiological concentrations, no significant increase in ssDNA breaks or 8-oxo-dG levels was detected compared to

controls (Figure 1A and B). It should be emphasized that HOCl is not considered to be a highly DNA-reactive agent and mainly targets proteins and lipids, before it reaches the nucleus (35). Early studies have shown that stimulated neutrophils can cause lipid peroxidation (36,37). Lipid peroxidation generates a complex variety of products among which are reactive electrophiles, such as epoxides and aldehydes (38). MDA is a major product of lipid peroxidation, capable of interacting with nucleic acid bases to form exocyclic DNA adducts, mainly  $\text{M}_1\text{dG}$  (26).  $\text{M}_1\text{dG}$  adducts have been shown to be promutagenic in mammalian cells and to induce frameshift



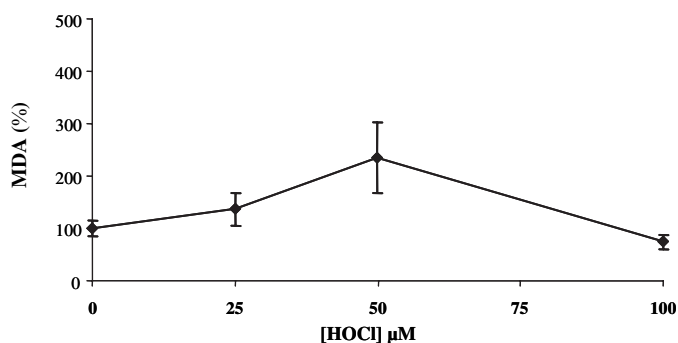
**Fig. 1.** DNA damage in A549 cells induced by HOCl *in vitro*. A549 cells were exposed to increasing concentrations of HOCl (0–100 µM) for 15 min at  $37^{\circ}\text{C}$ . (A) ssDNA breaks in A549 cells were monitored by comet assay and are expressed as mean  $\pm$  SEM % of control of duplicate determinations from two independent incubations. Comparison was drawn between treatment and unexposed control using Tail Moment (TM) parameters. A 250 µM  $\text{H}_2\text{O}_2$  was assessed as positive control ( $5873 \pm 3053$  % of control, data not shown). (B) 8-Oxo-dG was measured using HPLC-ECD and is expressed as mean 8-oxo-dG residues per  $10^6$  dG  $\pm$  SEM % of control of duplicate determinations from two independent incubations. A 250 µM  $\text{H}_2\text{O}_2$  was used as positive control ( $398 \pm 2$  % of control, data not shown). (C)  $\text{M}_1\text{dG}$  adducts were detected using  $^{32}\text{P}$ -postlabeling and expressed as mean  $\text{M}_1\text{dG}$  adducts per  $10^8$  nucleotides  $\pm$  SEM % of control of triplicate determinations from two independent incubations. Cell treatment with 250 µM  $\text{H}_2\text{O}_2$  gave  $345 \pm 9$  % of  $\text{M}_1\text{dG}$  adducts compared to control and was used as positive control (data not shown); \* $P < 0.05$ , \*\* $P < 0.01$  versus control.

mutations and base pair substitutions (39). In this way, HOCl may exert mutagenic effects indirectly via its attack on lipids. As a third genotoxic effect, we assessed the capacity of HOCl to induce M<sub>1</sub>dG adducts in A549 cells *in vitro*, being one of the major adducts resulting from lipid peroxidation (40). Results showed a significant (up to 3-fold,  $P < 0.01$ ) induction of M<sub>1</sub>dG by HOCl at 50  $\mu\text{M}$  (Figure 1C), independent from acute cytotoxicity. Clearly, such a rise in exocyclic M<sub>1</sub>dG adducts could be due to increased levels of MDA, but also to a decreased repair of these adducts, via the nucleotide excision repair pathway (41). The latter is in line with recent data from our laboratory, showing that neutrophil-derived HOCl inhibits nucleotide excision repair (42). To support the M<sub>1</sub>dG adduct data and to clarify if HOCl can induce aldehyde formation in lung epithelial cells, we measured MDA in A549 cells exposed to HOCl in terms of its reaction with thiobarbituric acid. Figure 2 illustrates higher MDA levels in cells treated with HOCl as measured by TBAR formation ( $P = 0.07$  at 50  $\mu\text{M}$  HOCl). This increase in MDA formation can be assigned to HOCl-induced lipid peroxidation. It should be noted, however, that also another pathway for aldehyde formation, independent of lipid peroxidation, may be important in the observed generation of MDA and associated M<sub>1</sub>dG adducts. Hazen *et al.* (43) have demonstrated that HOCl oxidizes amino acids to chloramines. The latter derivatizes loss of chloride and CO<sub>2</sub> to form imines; imines subsequently hydrolyse to aldehydes. Furthermore, M<sub>1</sub>dG adducts can also arise from the reaction of 2-deoxyguanosine with a DNA peroxidation product, namely base propenal, which is formed by oxidative attack of the sugar phosphate backbone. However, since we did not observe an increase of 8-oxo-dG, it is not clear whether direct oxidation of DNA by HOCl occurs in sufficient amounts to induce this alternative pathway leading to M<sub>1</sub>dG adduct formation.

In general, based on the genotoxic endpoints addressed in this study, these *in vitro* data suggest that the DNA-damaging effect induced by HOCl is mainly secondary via an oxidative attack on lipids and/or proteins.

#### Mutagenic effects of HOCl

To reveal whether M<sub>1</sub>dG adducts caused by HOCl are relevant to mutagenesis, the ability of HOCl to form mutations at the *HPRT* locus was investigated in A549 cells. First, long term survival after HOCl treatment was studied by determination of the clone-forming ability. Cells counted with dye exclusion



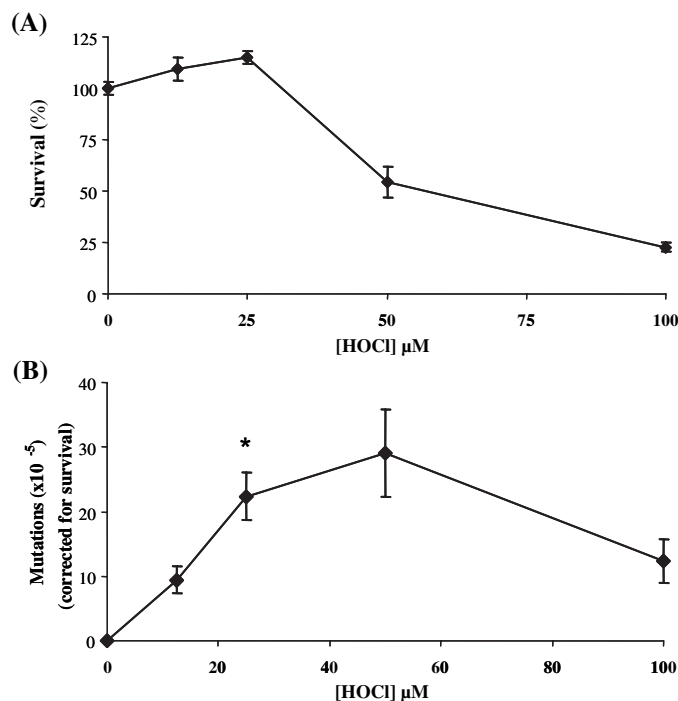
**Fig. 2.** HOCl induced lipid peroxidation in A549 cells. A549 cells were treated with increasing doses of HOCl (0–100  $\mu\text{M}$ ) for 15 min and formed TBARS were used as a measure for lipid peroxidation. Data are corrected for protein content in the lysate and are expressed as nmol MDA per mg protein % to control. Fe<sup>2+</sup> (10  $\mu\text{M}$ ) was used as positive control and values were 281% of MDA compared to control incubation (data not shown).

method and plated a few hours after treatment with increasing concentrations of HOCl showed a relative survival going from 100 to 25% at 100  $\mu\text{M}$  (Figure 3A). Figure 3B illustrates the mutant frequency corrected for this clonogenic survival after the expression time, showing a dose-dependent and statistically significant increase of the *HPRT* mutant frequency ( $P = 0.01$  at 25  $\mu\text{M}$  HOCl). The decrease in mutation frequency at 100  $\mu\text{M}$  HOCl is probably due to HOCl-induced cytotoxicity on a long term as observed by the clonogenic survival assay.

This is the first study to report on mutagenic effects of HOCl in mammalian cells. Both an increase in DNA lesions and the inhibition of DNA repair induced by HOCl may play a role in the process of *HPRT* gene mutation.

#### Neutrophil-induced DNA damage in epithelial cells: an *in vivo* mouse model of acute lung inflammation

Although the above *in vitro* findings may suggest M<sub>1</sub>dG adducts as being the most significant lesion underlying HOCl-induced mutagenicity in lung epithelial cells, the relevance with respect to the mechanism of HOCl-induced genotoxicity by neutrophils *in vivo* is unclear. Therefore, an LPS-induced acute lung inflammation mouse model expanded with a systemic depletion of neutrophils was used to elucidate the involvement of neutrophils and associated HOCl production in the induction of DNA damage in respiratory tract epithelial cells *in vivo*.



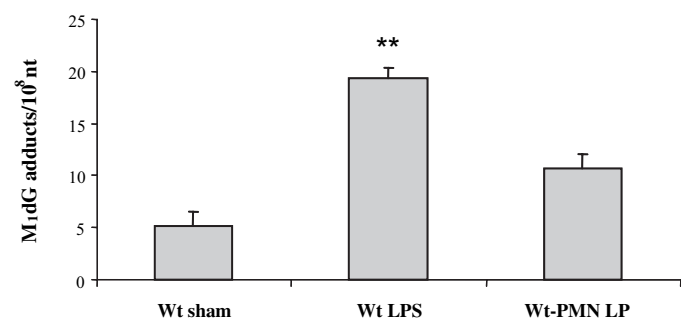
**Fig. 3.** Mutagenicity of HOCl. (A) Clonogenic survival assays were used to determine the sensitivity to 15 min acute treatment with HOCl in A549 cells. The mean values of three independent experiments are shown with SEM. (B) The number of *HPRT* mutants was determined via selection in medium containing 6-TG. Mutations were corrected for clonogenic survival and HOCl-induced mutation frequencies are expressed as mean *HPRT* mutations per  $10^5$  cells  $\pm$  SEM of three independent experiments. BPDE, a well-known mutagen, was assessed as positive control (3.0 and 521.7 *HPRT* mutations per  $10^5$  cells at 0.2 and 1  $\mu\text{M}$  BPDE, respectively). As only 25% of the A549 cells exposed to 100  $\mu\text{M}$  HOCl survived after 1 week of culturing, the associated mutation frequency data point was left out of statistical analysis, thus only the HOCl doses ranging from 0–50  $\mu\text{M}$  were taken in consideration; \* $P = 0.01$  versus control ( $P = 0.06$  at 50  $\mu\text{M}$  HOCl).

As extracellular MPO activity is indicative for the presence of activated neutrophils and serves as a surrogate marker for HOCl formation in the lung (33), MPO was assessed in BALF to verify neutrophil activation. Significant MPO activity was observed in the LPS-instilled mice ( $81.4 \pm 20.5$  mU/ml), whereas no MPO activity was detected in the sham group. Absent MPO activity in the neutrophil depletion group indicates absence of HOCl in these animals.

To reveal whether our *in vitro* observation, showing HOCl-induced M<sub>1</sub>dG adduct formation in alveolar epithelial cells, corresponds with the *in vivo* situation, we examined the DNA-damaging effects of neutrophils in pulmonary tissue of mice after i.t. LPS instillation. Similar as the *in vitro* finding on direct HOCl-induced genotoxicity, no direct oxidative DNA damage was found by 8-oxo-dG measurement in LPS-instilled mice ( $31.9 \pm 6.9$  8-oxo-dG/10<sup>6</sup> nucleotides) as compared to sham mice ( $25.3 \pm 4.3$  8-oxo-dG/10<sup>6</sup> nucleotides). This is in line with previous studies in which we were not able to find increased 8-oxo-dG levels in lungs from rats intratracheally treated with crystalline silica at dose levels that caused massive neutrophil influx into the airways (44). Although the baseline 8-oxo-dG levels reported here seem to be rather high, this was probably not due to spurious oxidation of the DNA as 8-oxo-dG measurement was performed in the presence of radical-free phenol, TEMPO and 8-hydroxyquinoline to prevent artificial 8-oxo-dG formation (22). In contrast, LPS treatment caused a significant increase in the formation of M<sub>1</sub>dG adducts in the mouse lung (~4-fold,  $P < 0.01$ ) (Figure 4). Moreover, a significant correlation was found between MPO activity in BALF and M<sub>1</sub>dG levels in epithelial cells (Pearson correlation = 0.67,  $P = 0.001$ ). This finding is in line with the above *in vitro* observations and suggests a link between neutrophil influx, MPO-mediated formation of HOCl and formation of M<sub>1</sub>dG adducts *in vivo*. The causal role of neutrophils and HOCl in this effect is further substantiated by the observation that depletion of circulating neutrophils causes a significant reduction in M<sub>1</sub>dG adduct levels in the LPS-treated mice.

## Conclusions

It has been proposed that ROS generated by activated inflammatory neutrophils may be involved in mutagenic processes and, hence, play a pivotal role in carcinogenesis. Focussing on HOCl as the major oxidant produced by neutrophils, this study is the first to report that HOCl induces mutations in lung epithelial cells indicating mainly an indirect genotoxic hazard of neutrophil-derived HOCl, by its attack on



**Fig. 4.** M<sub>1</sub>dG adduct levels in lung tissue after LPS exposure. Data are expressed as mean ± SEM of M<sub>1</sub>dG adducts per 10<sup>8</sup> nucleotides ( $n = 5-10$ ); \*\* $P < 0.01$  versus sham animals.

lipids and/or proteins producing MDA and forming M<sub>1</sub>dG adducts. Altogether, our data indicate that MPO-catalysed formation of HOCl should be considered as a significant source of neutrophil-induced DNA damage and possibly mutagenesis. Moreover, with regard to neutrophil infiltration in tumours, the release of HOCl may also contribute to genetic instability in tumour cells, thereby further stimulating tumour progression.

## Funding

Province of Limburg, The Netherlands; European Network of Excellence 'Environmental cancer, nutrition and individual susceptibility', sixth Framework programme (FP6) (FOOD-CT-2005-513943); Associazione Italiana per la Ricerca sul Cancro', Milan, Italy.

## Acknowledgements

The authors thank Yonca Güngör for linguistically reviewing the manuscript. Conflict of interest statement: None declared.

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Received on August 28, 2009; revised on October 7, 2009;  
accepted on October 9, 2009