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Monochloramine inhibits ultraviolet B-induced p53 activation and DNA repair response in human fibroblasts

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Abstract

Monochloramine (NH_2Cl) is one of the inflammation-derived oxidants, and has various effects on cell cycle, apoptosis and signal transduction. We studied the effects of NH_2Cl on DNA repair response induced by ultraviolet B (UVB) irradiation in normal human diploid fibroblasts, TIG-1. TIG-1 irradiated with 20 mJ/cm^2 UVB showed marked increase in thymine dimer, which decreased by about 50% after 24 h. This decrease in thymine dimer was significantly attenuated ($P<0.05$) by the pretreatment of NH_2Cl (200 μM), which indicated DNA repair inhibition. UVB induced p53 phosphorylation at Ser15, Ser20 and Ser37, and p53 accumulation, and NH_2Cl also inhibited these changes. Consequently, UVB-induced increase in the downstream effectors of p53, namely $p21^{Cip1}$ and *Gadd45a*, were almost completely inhibited by NH_2Cl. Immunoprecipitation study indicated that the association of p53 and MDM2, an E3 ubiquitin ligase for p53, did not change substantially by NH_2Cl and/or UVB. The phosphorylation of p53 (Ser15 and Ser37) by UVB is catalyzed by ATR (ataxia telangiectasia mutated and Rad3 related kinase), which works as DNA damage sensor, and ATR also phosphorylates checkpoint kinase 1(Chk1) at Ser345. NH_2Cl also inhibited the phosphorylation of Chk1 (Ser345). As UVB-induced DNA damage is repaired by nucleotide excision repair (NER) in human cells, these findings indicated that NH_2Cl inhibited NER through the inhibition of p53 phosphorylation and accumulation, and NH_2Cl probably impaired DNA damage recognition and/or ATR activation. NH_2Cl may facilitate carcinogenesis through the inhibition of NER that repairs DNA damages from various carcinogens.

Keywords: Monochloramine; p53; Nucleotide excision repair; Ultraviolet B; Inflammation; Carcinogenesis

1. Introduction

Chronic inflammatory diseases, such as interstitial pneumonia, *Helicobacter pylori*-induced gastritis, and chronic hepatitis, often develop malignant neoplasm [1]. Various causative mechanisms have been postulated for this inflammation-driven carcinogenesis, and one of such mechanisms is oxidative modification of biomolecules by reactive oxygen species (ROS) [2,3]. ROS and lipid peroxidation products may directly modify DNA base, which possibly produce carcinogenic mutations [2]. Besides these direct DNA base modifications, ROS may also promote carcinogenesis through epigenetic mechanism because ROS affect cellular signaling that regulates cell growth, apoptosis and DNA repair, which may eventually result in carcinogenesis [1,3].

Activated neutrophil is one of the most potent sources of ROS in the body. When neutrophils are activated, NADPH oxidase complex is assembled and produce superoxide anion [4,5]. The superoxide is converted to various oxidants such as hydrogen peroxide, hypochlorous acid, chloramines and hydroxyl radical in the reaction catalyzed by superoxide dismutase, myeloperoxidase and/or transition metals. Monochloramine (NH_2Cl) is a membrane-permeable oxidant produced in the reaction of HOCl and ammonium ion [6,7]. NH_2Cl preferentially oxidizes thiols and thioethers [8], and does not likely to modify DNA base. Nevertheless, NH_2Cl affects cell cycle, apoptosis, and response to cytokines through the

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oxidation of cell signal-related proteins such as retinoblastoma protein and IκB [9–12]. Hypochlorite and chloramines were reported to induce p53-dependent growth arrest [13]. In addition, N-chloramines are reported to be DNA repair inhibitors [14]. Thus, it is interesting to study if NH_2Cl has any epigenetic effects that possibly promote inflammation-induced carcinogenesis.

DNA damages activate several cellular responses, which include DNA repair, cell cycle arrest and apoptosis [15]. One of the most important proteins for DNA repair and cell cycle checkpoint is p53 tumor suppressor protein. The impairment of p53 function often associates with cancers. Indeed, more than half of the human cancers are reported to have functional abnormality in p53 protein [16]. Activation of p53 leads to cell cycle arrest, DNA repair and/or apoptosis [17]. Under normal conditions, p53 protein level is kept low by the continuous digestion by proteasome pathway [18,19]. Murine double minute 2 (MDM2) works as an E3 ubiquitin ligase for p53 [20]. When the cellular DNA is damaged, p53 is phosphorylated at multiple sites in vivo and accumulates in the cell [21]. Protein kinases ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3 related) work as DNA damage sensor [22], and phosphorylate downstream substrates such as checkpoint kinase 1 (Chk1) and Chk2, as well as p53 at Ser15 and Ser37 [23]. Chk2 and Chk1 can also phosphorylate p53 at Ser20, thereby enhancing its stability and activity [24,25]. In addition, Thr18 phosphorylation impairs the ability of p53 to bind MDM2, and promotes both accumulation and activation of p53 [26]. On the other hand, p53 phosphorylation at Ser46 is important in apoptosis induction [27,28]. Homeodomain-interacting protein kinase-2 and p38 kinase have been reported to phosphorylate Ser46 [27–29]. Activated p53 stimulates transcription of downstream effector proteins, namely $p21^{Cip1}$ and GADD45A. $P21^{Cip1}$ is a cyclin-dependent kinase inhibitor and arrest cell cycle to obtain sufficient time for DNA repair, while GADD45A is required for nucleotide excision repair [30].

In this paper, we studied the effects of NH_2Cl on DNA repair and DNA damage-induced accumulation and activation of p53. Our data indicated that NH_2Cl inhibited both DNA repair and DNA damage-induced accumulation of p53. The data imply that inflammation-derived oxidants may inhibit DNA repair through the inhibition of p53-dependent DNA repair/checkpoint function.

2. Materials and methods

2.1. Reagents

Antibodies were obtained as follows: thymine dimer from Kyowa Medex (clone: BM12, Tokyo, Japan), p53 from Novocastra (clone: DO-7, New Castle upon Tyne, UK) and Cell Signaling Technology (#9280, Beverly, MA), $p21^{Cip1}$ from Transduction Lab (clone: 70, BD Bioscience, Pharmingen, San Jose, CA), phosphorylated forms of p53, Chk1 and Chk2 from Cell Signaling Technology, and MDM2 from Santa Cruz Biotechnology (SMP14, Santa Cruz, CA). All other reagents are analytical grade or better. NH_2Cl (about 5 mM) was prepared just before experiments and the concentration was measured by UV absorption spectrum [31].

2.2. Cell culture and treatments

Human diploid fibroblast from fetal lung (TIG-1) was obtained from Health Science Research Resources Bank (Osaka, Japan) at population doubling=23, and used before the population doubling reaches 30. The cells were plated on collagen I-coated 35 mm dishes (Biocoat, collagen I, BD Falcon, Franklin Lakes, NJ), and the growth medium was Eagle's minimal essential medium (MEM-E) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin G (100 U/ml) and streptomycin (100 μg/ml). Cells were grown in a CO_2 incubator containing 5% CO_2 at 37 °C and used for experiments at 80–90% confluence.

Just before NH_2Cl pretreatment, the medium was replaced with MEM-E without FBS and antibiotics (1.5 ml/dish), and then NH_2Cl was added to 200 μM of final concentration, and incubated for 10 min at 37 °C. When the oxidant concentration was measured by 5-thio-2-nitrobenzoate assay [31,32] after the 10 min of incubation, more than 90% of added NH_2Cl disappeared from the medium and probably reacted with cells and medium components (data not shown). Immediately after NH_2Cl treatment, the cells were irradiated with ultraviolet B (UVB) lamp (ATTO, HP-6M) that has a peak wavelength of 312 nm, at 400 $\mu W/cm^2$ for 50 s, which amounted to 20 mJ/cm^2. The UVB intensity was measured by UVX radiometer equipped with UVX31 detector (UVP Inc., Upland, CA). Preliminary experiment showed that this UVB dose did not induce cell death and suitable to study DNA repair. After the treatments, the medium was replaced with a fresh growth medium, and incubated for the indicated times in a CO_2 incubator. Cells were harvested using 0.25% trypsin and 0.02% EDTA solution (Life technologies, Carlsbad, CA).

2.3. Measurement of thymine dimer

Thymine dimer was semi-quantified using slot blot analysis as described previously [33]. Briefly, DNA was extracted from the cells using DNeasy Tissue Kit (Qiagen, Valencia, CA) following manufacturer's instruction, and DNA concentration was measured by a spectrophotometer. For each samples, 200 μg of DNA was denatured by heating at 100 °C for 2 min, cooled quickly and blotted on a nylon membrane (Hybond N+, Amersham Piscataway, NJ) using Minifold II blotting apparatus (Schleicher and Schuell, Dassel, Germany). The nylon membrane was baked at 80 °C for 2 h, and thymine dimer was detected using anti-thymine dimer antibody and enhanced chemiluminescence method (Nacalai Tesque, Kyoto, Japan).

2.4. Western blot analysis

The harvested cells were washed with ice-cold PBS, and cellular proteins were extracted in a lysis buffer (20 mM HEPES (pH 7.5), 40 mM β-glycerophosphate, 20 mM NaF, 1 mM Na_3VO_4, 20 mM p-nitrophenyl phosphate, 1 mM dithiothreitol, 420 mM NaCl, 0.1%(v/v) Nonidet-P40, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Protein concentrations were measured by Bradford method [34], and the same amounts of protein were separated by a 10–20% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Immunoreactive proteins were detected using the enhanced chemiluminescence system.

2.5. Immunoprecipitation

Anti-MDM2 antibody (50 μg) was covalently bound to protein G agarose beads (50 μl) using Seize X protein G immunoprecipitation kit (PIERCE, Rockford, IL) following manufacturer's instruction. The cell lysate containing 120 μg protein (approximately 40 μl) was first diluted to make the salt concentration about 150 mM, and then incubated with 5 μl of antibody-bound beads for 6 h at 4 °C. After centrifugation, the beads and the supernatant were collected separately. The beads were washed three times with D-PBS, then added with 20 μl of SDS-PAGE sample buffer and boiled in a water bath for 3 min. The supernatant was concentrated using centrifugal microconcentrator (Centricut W-10, KURABO, Osaka, Japan), then the protein concentration was measured, and 20 μg of protein was analyzed by SDS-PAGE. The cell lysate without immunoprecipitation (20 μg protein) was also analyzed at the same time.

2.6. Quantitative PCR

Total RNA was extracted from the cells using RNeasy mini Kit (Qiagen) following manufacturer's instruction, and the obtained RNA concentration was measured by a spectrophotometer. From 55 ng of RNA samples, cDNA was prepared using SuperScript First-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) following manufacturer's instruction. Quantitative PCR was performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and Lightcycler (Roche, Basel, Switzerland) with the following settings: denature: 94 °C for 5 s, anneal: 55 °C for 10 s, extension: 72 °C for 10 s, data acquisition at 85 °C for 1 s, 40 cycles). The primer sequences were *Gadd45a* -forward: 5′-agaagaccgaaaggatggat-3′, *Gadd45a* -reverse: 5′-atctgcagagccacatctct-3′, *GAPDH*-forward: 5′-ttggtatcgtggaaggactc-3′, *GAPDH*-reverse: 5′-tagaggcagggatgatgttc-3′.

2.7. Cell cycle analysis

The cell cycle was studied by measuring the DNA content by the PI staining method [35], except that the samples were supplemented with RNase A (200 μg/ml). Samples were analyzed by a flow cytometer (FACSCalibur, Becton-Dickinson, Franklin Lakes, NJ, USA). Data were collected from a morphometrically homogeneous population, which contains more than 80% of the total events.

2.8. Image analysis and statistical analysis

For slot blot and Western blot images, each band densities were measured using Image J software (http://rsb.info.nih.gov/ij/), and expressed as % of the sum of total band densities. Group means were compared using Student's *t* test. Analysis of variance with Tukey's test was also used for multiple comparison using Statcel QC software (OMS publishing Inc., Saitama, Japan). The *P* values less than 0.05 were considered as significant.

3. Results

3.1. NH_2Cl inhibited DNA repair from UVB-induced damage

UVB (20 mJ/cm^2) irradiation substantially increased thymine dimer in TIG-1 cells. Without NH_2Cl treatment, this thymine dimer was removed from DNA with time, and after 24 h of UVB irradiation, the band density of thymine dimer decreased by approximately 50% of the time = 0 h samples (Fig. 1). When the cells were treated with NH_2Cl (200 μM) before UVB irradiation, the removal of thymine dimer from DNA was significantly attenuated, in which the thymine dimer band density at 24 h was about 80% of the time = 0 h samples (Fig. 1). The result indicated that NH_2Cl-treated fibroblasts were defective in DNA repair.

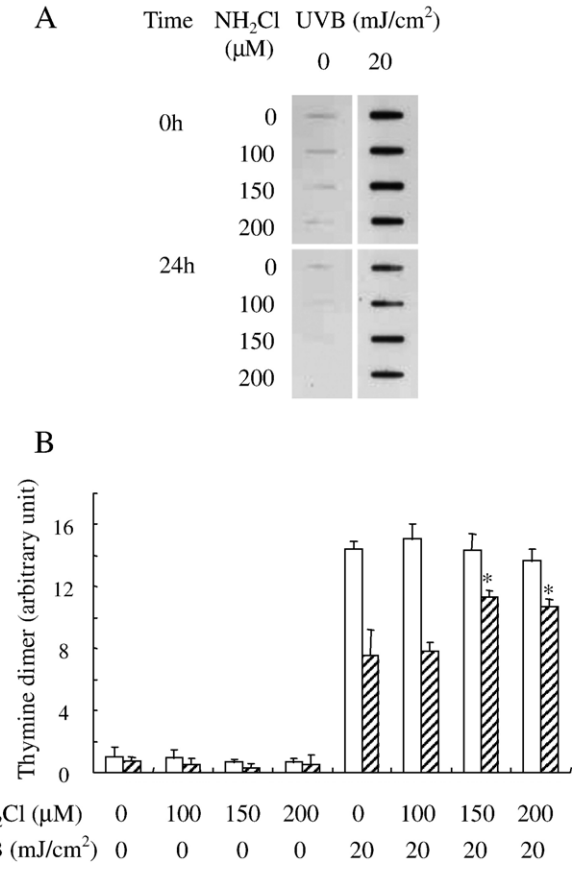

Fig. 1. NH_2Cl inhibited DNA repair from UVB-induced damage. TIG-1 were treated with indicated concentrations of NH_2Cl for 10 min at 37 °C, and then irradiated with 20 mJ/cm^2 UVB. Then, the cells were collected immediately or after 24 h of incubation. Heat-denatured DNA (200 μg) was blotted on a nylon membrane, and thymine dimer was detected by anti-thymine dimer antibody. (A) Representative slot blot image. (B) Optical densities were measured with Image J software and are expressed as % of the sum of total band densities. Open bars: 0 h, hatched bars: 24 h. Mean ± S.D. from three independent experiments. *Significantly higher than NH_2Cl = 0 and UVB = 20 mJ/cm^2 samples.

3.2. NH_2Cl inhibited UVB-induced p53 accumulation and phosphorylation at Ser15, 20 and 37

UVB irradiation induced phosphorylation of p53 at Ser15, Ser20 and Ser37, and accumulation of the p53 protein (Fig. 2). The Ser15 phosphorylation increased slightly at 2 h after UVB irradiation, and increased further at 6 h. In parallel with this phosphorylation, p53 protein accumulated in the UVB-irradiated cells. Phosphorylation at Ser20 and Ser37 were detectable at 6 h after UVB irradiation. Thr18 phosphorylation was not detectable (data not shown). NH_2Cl treatment almost completely abolished UVB-induced p53 phosphorylation at these three sites and inhibited the p53 accumulation at 2 and 6 h after UVB irradiation. Although NH_2Cl treated cells showed a little more dense p53 band at 6 h, the increase was not statistically significant. In addition, NH_2Cl also increased Ser46 phosphorylation of p53 (Fig. 2A). UVB at 20 mJ/cm^2 showed no effect on Ser46 phosphorylation.

3.3. NH_2Cl inhibited $p21^{Cip1}$ expression

Cyclin dependent kinase inhibitor $p21^{Cip1}$ is one of the downstream effector molecules of p53. As reported previously, p53 accumulation by UVB irradiation resulted in $p21^{Cip1}$ increase in control fibroblasts [36] (Fig. 3). In the NH_2Cl-treated cells, however, this UVB-induced $p21^{Cip1}$ accumulation did not occur. On the contrary, NH_2Cl treatment decreased $p21^{Cip1}$ at 2 and 6 h, regardless of UVB irradiation.

3.4. Gadd45a mRNA expression was also suppressed by NH_2Cl

GADD45A is another downstream effector of p53. When *Gadd45a* mRNA was quantified by real-time PCR, UVB

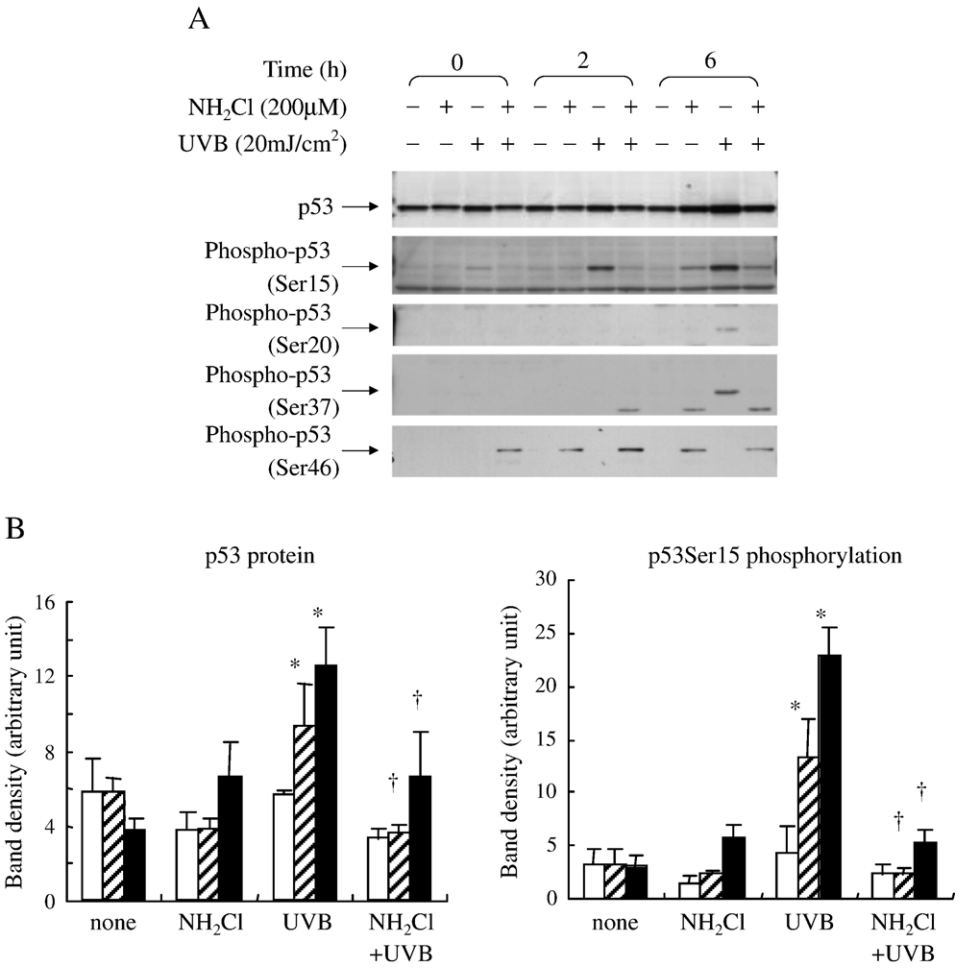

Fig. 2. Accumulation and phosphorylation status of p53 after NH_2Cl /UVB treatment. TIG-1 was treated with 200 μM NH_2Cl for 10 min at 37 °C, and then irradiated with 20 mJ/cm^2 UVB. Then, the medium was replaced with fresh medium, and incubated for the indicated times. Accumulation and the phosphorylation status of p53 were studied by the Western blotting method. (A) Representative Western blot image. (B) Open bars: 0 h, hatched bars: 2 h, closed bars: 6 h. Mean ± S.D. from three independent experiments. *Significantly higher than "none" samples. †Significantly decreased from "UVB" samples.

irradiation significantly increased its amount (Fig. 4). Again, NH_2Cl treatment almost completely inhibited the UVB-induced expression of *Gadd45a* mRNA. *GAPDH* mRNA levels were also measured as internal control. The *GAPDH* mRNA levels showed no remarkable difference between each samples (data not shown), and the *Gadd45a* expression was shown as the ratio of *Gadd45a*/*GAPDH*.

3.5. NH_2Cl decreased MDM2 protein, and most of the p53 did not co-immunoprecipitate with MDM2

MDM2 works as an E3 ubiquitin ligase for p53, and its expression is under the transcriptional control of p53 [37]. MDM2 protein expression was decreased by NH_2Cl treatment (Fig. 5). MDM2 and p53 association was studied by co-immunoprecipitation method at 2 h after NH_2Cl /UVB treatment. Fig. 6 showed that only a slight fraction of the p53 was co-immunoprecipitated with MDM2, and most of the p53 remained in the supernatant. UVB irradiated samples showed highest amount of p53 co-immunoprecipitated with MDM2, however, it should be noted that supernatant p53 amount was also highest in this group. Thus, it appeared that the ratio of p53 that was associated with MDM2 was not different substantially between each groups.

3.6. NH_2Cl inhibited UVB-induced Chk1, Chk2 phosphorylation

In addition to p53 phosphorylation, UVB irradiation also evokes other protein phosphorylation, namely Chk1 (Ser345) and Chk2 (Ser19) [15]. Therefore, the effects of NH_2Cl on Chk1 and Chk2 phosphorylation were studied. UVB irradiation increased phosphorylation of Chk1 (Ser345) and Chk2 (Ser19) at 2 h and/or 6 h (Fig. 7). Interestingly, Chk1 (Ser345) and Chk2 (Ser19) phosphorylation were also suppressed significantly by NH_2Cl-treatment.

NH_2Cl induced a slight decrease in cell viability, nevertheless, more than 80% of the cells were viable after 24 h of NH_2Cl treatment (Fig. 8). UVB at 20 mJ/cm^2 did not result in any loss of viability.

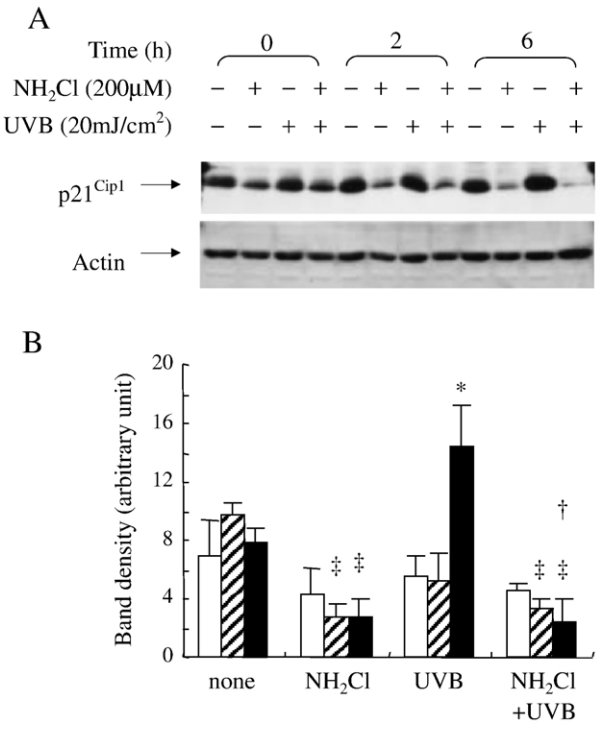

Fig. 3. Expression of $p21^{Cip1}$ protein by UVB and its inhibition by NH_2Cl. TIG-1 cells were treated as described in Fig. 2 legend and cultured for the indicated times. Expression of the $p21^{Cip1}$ was studied by the Western blotting method. (A) Representative Western blot image. Actin expression indicated the same protein load of each samples. (B) Open bars: 0 h, hatched bars: 2 h, closed bars: 6 h. Mean±S.D. from three independent experiments. *Significantly higher than "none" samples. †Significantly decreased from "UVB" samples. ‡Significantly decreased from "none" samples.

Cell cycle distribution was measured 24 h after NH_2Cl and/or UVB treatment. NH_2Cl treatment resulted in slight decrease in G_2/M population, and increase in G_0/G_1 phase cells (Fig. 9). No significant change was observed in S phase cells. UVB at 20 mJ/cm^2 showed no significant change in the cell cycle status.

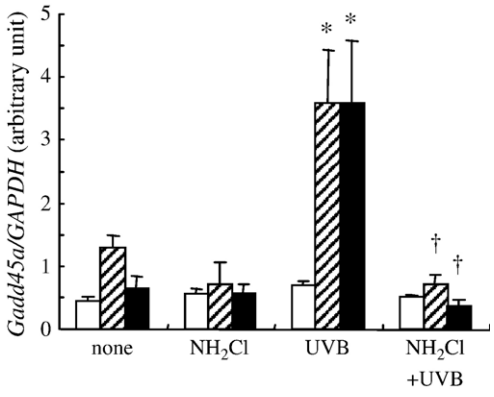

Fig. 4. UVB-induced *Gadd45a* mRNA expression and its inhibition by NH_2Cl. TIG-1 cells were treated as described in Fig. 2 legend and cultured for the indicated times. RNA samples were prepared and *Gadd45a* mRNA was quantified by real time PCR as described in Materials and methods. *GAPDH* mRNA was also quantified from the same sample as an internal control, and the data were shown as *Gadd45a*/*GAPDH* (arbitrary unit). Open bars: 0 h, hatched bars: 2 h, closed bars: 6 h. Mean±S.D. from three independent experiments. *Significantly higher than "none" samples. †Significantly decreased from "UVB" samples.

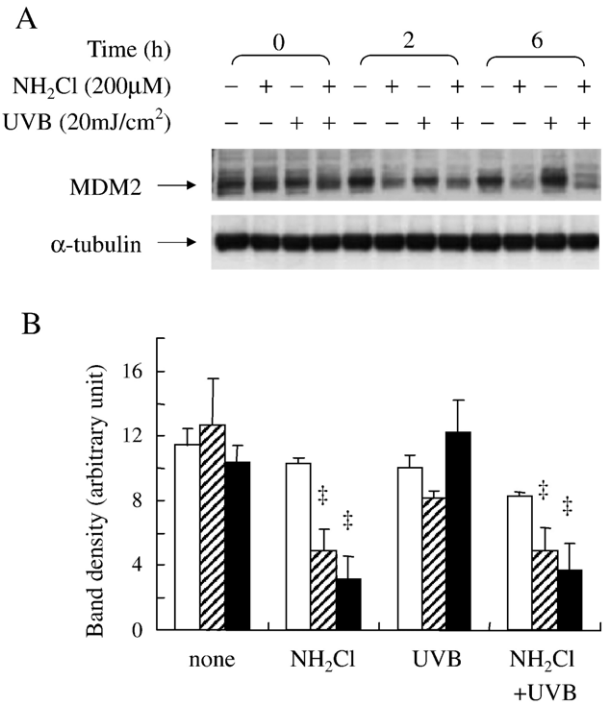

Fig. 5. Expression of MDM2 and its decrease by NH_2Cl. TIG-1 cells were treated as described in Fig. 2 legend and cultured for the indicated times. Expression of MDM2 was studied by the Western blotting method. (A) Representative Western blot image. Tubulin expression indicated the same protein load of each samples. (B) Open bars: 0 h, hatched bars: 2 h, closed bars: 6 h. Mean±S.D. from three independent experiments. ‡Significantly decreased from "none" samples.

4. Discussion

In this paper we demonstrated that DNA repair from UVB-induced damage was attenuated by NH_2Cl. UVB irradiation enhanced p53 phosphorylation at Ser15, Ser20 and Ser37, and

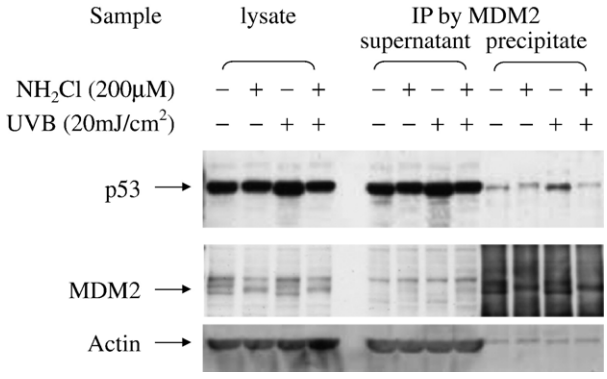

Fig. 6. Association of MDM2 and p53. TIG-1 cells were treated as described in Fig. 2 legend and cultured for 2 h. The cell lysate was immunoprecipitated with anti-MDM2 antibody, and the precipitated protein as well as the supernatant protein was analyzed by Western blot using the indicated antibodies. Representative Western blot image from three experiments. IP: immunoprecipitation.

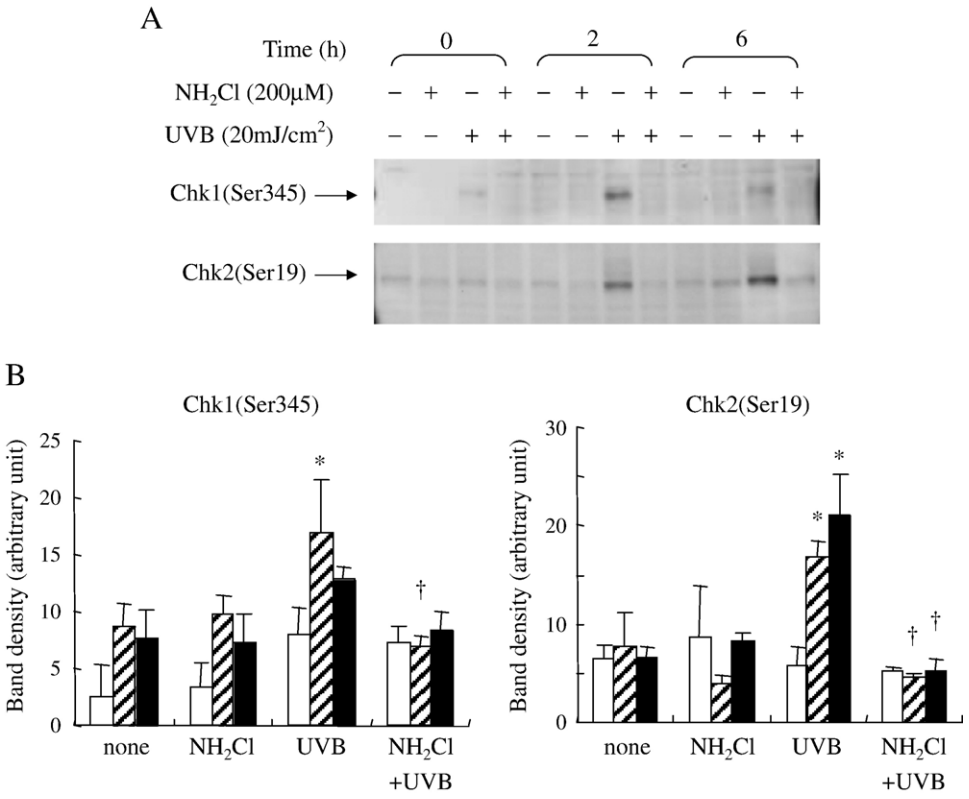

Fig. 7. UVB-induced phosphorylation of Chk1 (Ser345) and Chk2 (Ser19), and their inhibition by NH_2Cl. TIG-1 cells were treated as described in Fig. 2 legend and cultured for the indicated times. Phosphorylation of Chk1 (Ser345) and Chk2 (Ser19) was studied by the Western blotting method. (A) Representative Western blot image. (B) Open bars: 0 h, hatched bars: 2 h, closed bars: 6 h. Mean±S.D. from three independent experiments. *Significantly higher than "none" samples. †Significantly decreased from "UVB" samples.

these phosphorylations were inhibited by NH_2Cl. In addition, UVB-induced p53 accumulation was also inhibited by NH_2Cl. Consequently, the downstream effectors of p53, namely $p21^{Cip1}$ and *Gadd45a*, were not induced in NH_2Cl-treated cells. MDM2 is also under the transcriptional regulation of p53, and NH_2Cl decreased MDM2 and $p21^{Cip1}$ protein levels. This result may suggest that p53 transcriptional activity was defective in NH_2Cl-treated cells.

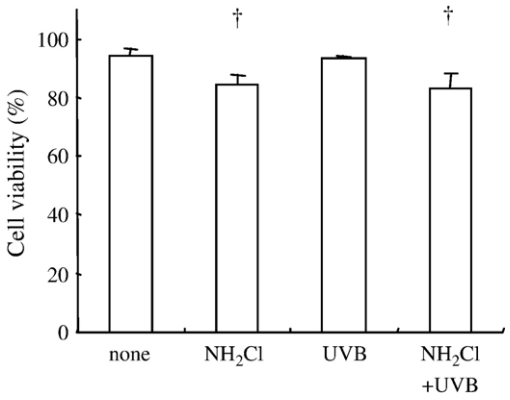

Fig. 8. Cell viability at 24 h after NH_2Cl /UVB treatment. TIG-1 cells were treated as described in Fig. 2 legend and cultured for 24 h. Cell viability was measured by dye-exclusion method using propidium iodide, and analyzed by a flow cytometer. Mean±S.D. from three independent experiments. †Significantly decreased from "none" samples.

Posttranslational modifications of p53, including phosphorylation of various Ser/Thr residues and acetylation, regulate the stability and transcriptional activity of p53 [38]. One important mechanism of stabilizing p53 is the interaction with MDM2, a major E3 ubiquitin ligase for p53. It is reported that Thr18

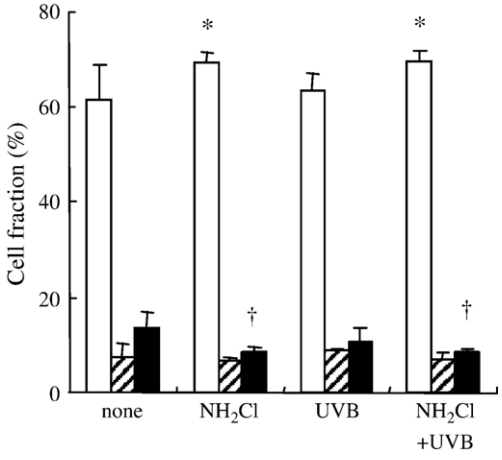

Fig. 9. Cell cycle distribution at 24 h after NH_2Cl /UVB treatment. TIG-1 cells were treated as described in Fig. 2 legend and cultured for 24 h. Cells were stained with propidium iodide supplemented with Triton X-100, and their DNA contents were measured by a flow cytometer. G_0/G_1 (open bars), S (hatched bars) and G_2/M (closed bars) phase cells were counted and expressed as % of total events. Mean±S.D. from three independent experiments. *Significantly higher than "none" samples. †Significantly decreased from "none" samples.

phosphorylation of p53 significantly decrease the binding affinity of p53 and MDM2 [39,40]. However, other p53 phosphorylation sites, such as Ser15, Ser20 and Ser37, are also important for p53 stabilization [41–43]. Although we tried to detect Thr18 phosphorylation, it was not detectable (data not shown). Since NH_2Cl inhibited p53 phosphorylation at Ser15, Ser20 and Ser37, it is likely that p53 stabilization did not occur successfully, and p53 was not accumulated in NH_2Cl -treated cells. We also studied the association of MDM2 and p53, which showed that most of the p53 did not co-immunoprecipitate with MDM2 in all groups. The result may suggest that MDM2 and p53 does not exist as a stable complex, and once the complex is formed, it may be quickly ubiquitinylated and digested by proteasome.

UVB-induced p53 phosphorylation at Ser15 and Ser37 are catalyzed by ATR [22,23], and these phosphorylation was inhibited by NH_2Cl. ATR works as DNA damage sensor and phosphorylates Chk1, Chk2, as well as p53. Chk1 and Chk2 work downstream of ATM/ATR, and Chk2 phosphorylates p53 (Ser20). Our results showed that Chk1 (Ser345) and Chk2 (Ser19) phosphorylation were also inhibited by NH_2Cl at 2 and 6 h after UVB. Both of the sites are known to be phosphorylated by ATM/ATR kinase. Thus, the results suggested that NH_2Cl inhibited DNA damage recognition by ATR and/or ATR kinase activity, and this possibility should be studied further.

Interestingly, NH_2Cl enhanced Ser46 phosphorylation. Phosphorylation at Ser46 has been reported to be catalyzed by homeodomain-interacting protein kinase-2 and p38 kinase, and Ser46 phosphorylation stimulates apoptosis through the induction of various genes that contribute to apoptosis [27–29]. The result indicated that p53-mediated DNA repair and apoptosis were independently regulated by different kinases, and that NH_2Cl suppressed DNA repair, while stimulated apoptosis. Consistently, NH_2Cl treatment resulted in a slight decrease in viability (Fig. 8), which may be a result of apoptosis induction by NH_2Cl. The result is consistent with our previous works, which indicated that NH_2Cl induced apoptosis in leukemic cells through cytochrome *c* release from mitochondria [44,45].

Inhibition of the p53 accumulation probably impaired nucleotide excision repair (NER), because *p53* knockout animal is reported to show defect in global genomic repair, one of the two sub-pathways of NER [30]. P53 regulates various genes, and some of them, such as *Gadd45a*, *XPC* and *p48XPE* [46,47], probably participate in damage recognition, thereby execute the initial step of NER process [48,49]. Thus, NH_2Cl probably inhibited NER through the inhibition of p53 activation and expression of the downstream effector proteins. In addition to NER, NH_2Cl may also affect base excision repair, because it is reported that p53 also participate in base excision repair [49,50].

One important question is whether the observed inhibition of DNA repair is only applicable to UVB-induced damage, or it is also the case with the DNA damages induced by other genotoxic stress, such as carcinogenic chemicals. UVB irradiation causes rather bulky DNA damage, such as cyclobutane pyrimidine dimer (thymine dimer) and 6,4 photoproducts. These bulky DNA damages are repaired mostly by NER [15]. In this respect, many carcinogenic stresses, such as chemicals, also cause bulky DNA damages, which are typically repaired by NER [15,51]. Therefore, NH_2Cl is likely to inhibit DNA repair from various carcinogens. As NH_2Cl inhibited the expression of *Gadd45a* and $p21^{Cip1}$, NH_2Cl-treated cells may behave as if they have no functional p53. This is an interesting model in which p53 function is lost under certain oxidative stress without any mutation in the *p53* gene itself. Oxidative stress may transiently suppress p53 response, thereby allowing DNA damage to persist, which may eventually result in carcinogenesis.

In contrast to our present work, previous report showed that HOCl and chloramines increased p53 and $p21^{Cip1}$ protein in human fibroblast, which resulted in growth arrest [13]. As our chloramine concentration was substantially higher, p53 transcriptional activity might be suppressed in our study. Despite the decrease in $p21^{Cip1}$(Fig. 3), NH_2Cl apparently induced cell cycle arrest (Fig. 9). Our previous work showed that oxidation of retinoblastoma protein by NH_2Cl induced cell cycle arrest [9], and probably a similar mechanism is working in TIG-1 cells.

In this experiment, NH_2Cl was added to the cells in MEM-E. When chloramine is added to a medium containing amino compounds, chlorine transfers to form other chlorinated oxidants [52]. The reaction depends on the relative concentration of amino compounds. Thus, the observed effects might be a result from NH_2Cl and other chlorinated oxidants. Under physiological conditions, ammonium ion is especially abundant in large intestine and *Helicobacter pylori*-infected stomach [53,54]. Thus, inflammatory diseases in these organs, such as inflammatory bowel disease or *H. pylori*-gastritis, are particularly predisposed to NH_2Cl production. It is interesting to note that malignant transformation is a frequent complication of these diseases [55]. When NH_2Cl is produced in these diseases, it may inhibit NER, and persistent DNA damage may lead to a permanent genomic alteration, which eventually results in carcinogenesis.

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