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TYROSINE-NITRATION OF CASPASE 3 AND CYTOCHROME C DOES NOT SUPPRESS APOPTOSIS INDUCTION IN SQUAMOUS CELL CARCINOMA CELLS

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The influence of tyrosine nitration of cytochrome c and caspase 3 on apoptosis induction was investigated in an established squamous carcinoma cell line, OSC-4. The intracellular NO and O_2^- levels were increased up to about 110–120% and 140–180% of the control levels, respectively, after the treatment of OSC-4 cells with 5-FU (100 μg/ml), PLM (10 μg/ml), CDDP (10 μg/ml), or γ-rays (20 Gy). The treatment of OSC-4 cells with $ONOO^-$ (1 mM) and the above anticancer agents induced tyrosine nitration of 14, 32 kDa protein among others and nitration of tyrosine residues of cytochrome c and caspase 3 was identified by the Western blotting of immunoprecipitates obtained by antibodies to these proapoptotic proteins. When cytochrome c and procaspase 3 were treated with $ONOO^-$, tyrosine nitration was increased in a $ONOO^-$-dose dependent manner. Tyrosine nitration of cleaved (17 kDa) caspase 3, however, was not induced by $ONOO^-$. Procaspase 3 in the cytosol of HeLa cells was activated by the addition of $ONOO^-$-treated as well as $ONOO^-$-untreated cytochrome c. In addition, cleavage of ICAD and PARP were not suppressed in OSC-4 cells by pretreatment with $ONOO^-$. Activity of cleaved caspase 3 was not suppressed at low concentrations or by treatment with $ONOO^-$ or NO donors, SIN-1 and SNP. Furthermore, apoptosis of OSC-4 cells by the anticancer agents was not suppressed by $ONOO^-$. In conclusion, these results suggest that nitration of tyrosine residues of cytochrome c and procaspase 3 is induced by chemoradiotherapy but their nitration does not suppress cancer cell apoptosis.

Key words: *tyrosine-nitration; caspase 3; cytochrome c; apoptosis; squamous cell carcinoma cells; anticancer agents*

Successful anticancer therapy with chemicals and radiation strongly depends on the strategy for differentiation and apoptosis induction of cancer cells. ROS induce apoptotic signals in cancer cells and apoptosis induction by anticancer drugs and γ-rays is closely associated with intracellular ROS generation.[1–7] Apoptotic signals are transduced mainly through 2 pathways, one of which is originates from the mitochondria and the other is the Fas-associated route.[8–10] The release of the main mitochondrial proapoptotic protein, cytochrome c, is under the regulation of ROS.[11,12] In addition to the generation of ROS, NO is also generated in anticancer drug- and γ-ray-treated cancer cells and reaction of ROS with NO is allowed in a favorable circumstance.[13,14] The reactant $ONOO^-$ vigorously damages cells by impairment of proteins and DNA.[15–19] Inactivation of the proapoptotic proteins by $ONOO^-$ is therefore probable in chemoradiotherapy of cancers although the apoptosis-inducing activity of $ONOO^-$ has been demonstrated in multiple kinds of cancer cells.[20,21]

NO reacts with molecular oxygen (O_2), O_2^- and transitional metals, yielding nitrogen dioxide (NO_2), $ONOO^-$ and metal-nitrosyl adducts, respectively.[22–24] These reactants are highly reactive and exhibit a variety of physiological activities.[25] In many disorders including inflammatory diseases, the neurotoxicity of stroke, Alzheimer's disease, Parkinson's disease and multiple sclerosis, the highly reactive nitrogen chemicals, especially $ONOO^-$, are deeply associated with the pathological degeneration of the tissues although the half life of $ONOO^-$ is very short, less than one second in the physiological condition.[26,27] $ONOO^-$ has a potential to cause DNA-strand breaks, lipid peroxidation and diverse modification of cellular molecules such as oxidation of thiol and methionine and nitration of tyrosine and tryptophane.[28] In these modifications, tyrosine nitration has been extensively explored with the aim of analysis of the cytotoxic mechanism of $ONOO^-$.[15–17,29]

A few investigators reported nitration of cytochrome c and s-nitrosylation of caspase 3 by $ONOO^-$, but they did not examine the influence of nitration on the catalytic and proteolytic activities of these proteins.[30–32] Cytochrome c and procaspase 3 have 4 and 10 tyrosine residues, respectively. $ONOO^-$ inhibits the electron transport in mitochondria and $ONOO^-$ induces nitration of Tyr34 of manganese-superoxide dismutase in the mitochondrial matrices.[33] It is likely that when the tyrosine residues of cytochrome c and procaspase 3 are nitrated and these proapoptotic proteins are inactivated, the generation of $ONOO^-$ in cancer cells is disadvantageous for apoptosis induction. We report the nitration of cytochrome c and procaspase 3 by $ONOO^-$ and the influence of their tyrosine nitration on apoptosis using a squamous cell carcinoma cell line.

MATERIAL AND METHODS

Cells

HeLa cells, a cell line established from a cervical carcinoma and OSC-4 cells, an oral squamous cell carcinoma cell line that has a silent point mutation of the p53 gene at codon 174 (AGG→AGA),[34] were used in our study. They were cultured in DMEM supplemented with 10% FBS. In every examination, the subconfluent phases of the monolayered cultured cells were used.

Preparation of $ONOO^-$

Peroxynitrite was prepared by the reaction of equivalent volume of H_2O_2 and 2-methoxyethyl nitrite under basic condition as described by Lin *et al.*[35] Peroxynitrite was produced by mixing 1.5 ml of H_2O_2 (0.1 M) with 1.5 ml of 2 N sodium hydroxide and 7 ml of distilled H_2O. To the mixture, 0.15 ml (1.6 mM) of 2-methoxyethyl nitrite was added at room temperature. The yielded $ONOO^-$ was quantified by UV spectrum of the solution 15 min after the mixing (λ_{max} = 302 nm: ϵ = 1,670 ± 50 l/mol cm). The light yellow solution of $ONOO^-$ (150–200 mM) was stored at −80°C for an extended time. Aliquots of $ONOO^-$ were monitored spectroscopically at 302 nm to accurately determine the concentration before and after each experiment.

Abbreviations: CAD, caspase-activated DNase; CDDP, cis-diamminedichloroplatinum; 5-FU, 5-fluorouracil; H_2O_2, hydrogen peroxide; ICAD, inhibitor of CAD; NO, nitric oxide; $ONOO^-$, peroxynitrite; O_2^-, superoxide; PARP, poly(ADP-ribose)polymerase; PLM, peplomycin; pNA, *p*-nitroaniline; ROS, reactive oxygen species; SIN-1, 3-morpholinosydronimine; SNP, sodium nitroprusside.

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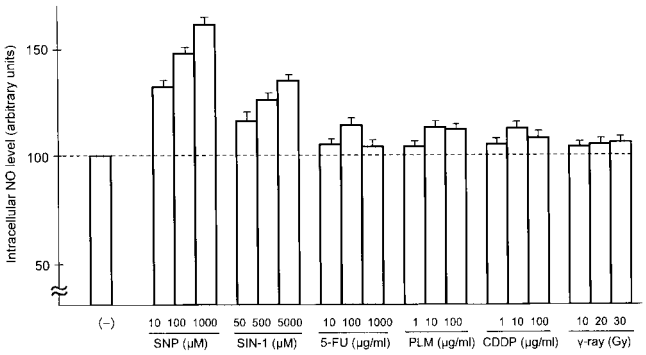

FIGURE 1 – Intracellular NO levels in OSC-4 cells treated with NO donors or anticancer agents. OSC-4 cells were treated with each indicated agent for 1 hr at 37°C. Then, the harvested cells were incubated in the presence of 10 μM of 4.5 diaminofluorescene diacetate for 15 min at room temperature. After being washed with PBS, the cells were examined by flow cytometry. The examination was triplicate and the values shown are the mean relative fluorescence intensities, indicating that the intensity in the nontreated OSC-4 cells was 100. Scale bar = mean ± 1 SD.

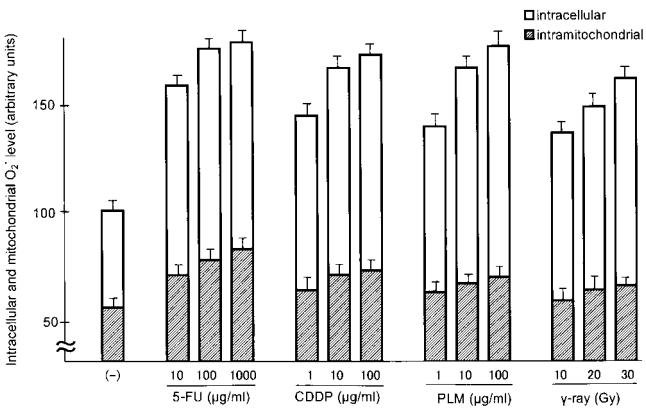

FIGURE 2 – Intracellular and intramitochondrial O_2^- levels in OSC-4 cells treated with anticancer agents. Non-fractionated OSC-4 cells or mitochondria extracted from OSC-4 cells were treated with each indicated agent and cultured for 1 hr at 37°C. After treatment with hydroethidine (2 μM) for 15 min, the O_2^- level was determined by flow cytometry. Scale bar = mean ± 1 SD of the triplicates under the indication of the mean fluorescence in the non-treated cells and mitochondria as 100.

Treatment of OSC-4 cells

OSC-4 cells cultured in 100 mm dishes were treated with 5-FU (100 μg/ml, Kyowa Hakko Co., Tokyo), CDDP (10 μg/ml, Nihon Kayaku Co., Tokyo) or PLM (10 μg/ml, a bleomycin derivative, Nihon Kayaku Co.) for 6–48 hr at 37°C in the presence or absence of 150 or 1,000 μM peroxynitrite. The cells were then harvested and used for the examination. After irradiation (20 Gy) with ^{137}Cs, OSC-4 cells were cultured as described above and harvested for the examination.

Assay of intracellular NO and O_2^- level

Intracellular NO and O_2^- levels were assayed by flow cytometry using 4,5-diaminofluorescein diacetate (10 μM) and hydroethidine (2 μM) purchased from Sigma (St. Louis, MO) and Molecular Probes (Eugene, OR). The intramitochondrial O_2^- level was also assayed by flow cytometry using hydroethidine with mitochondrial fraction.

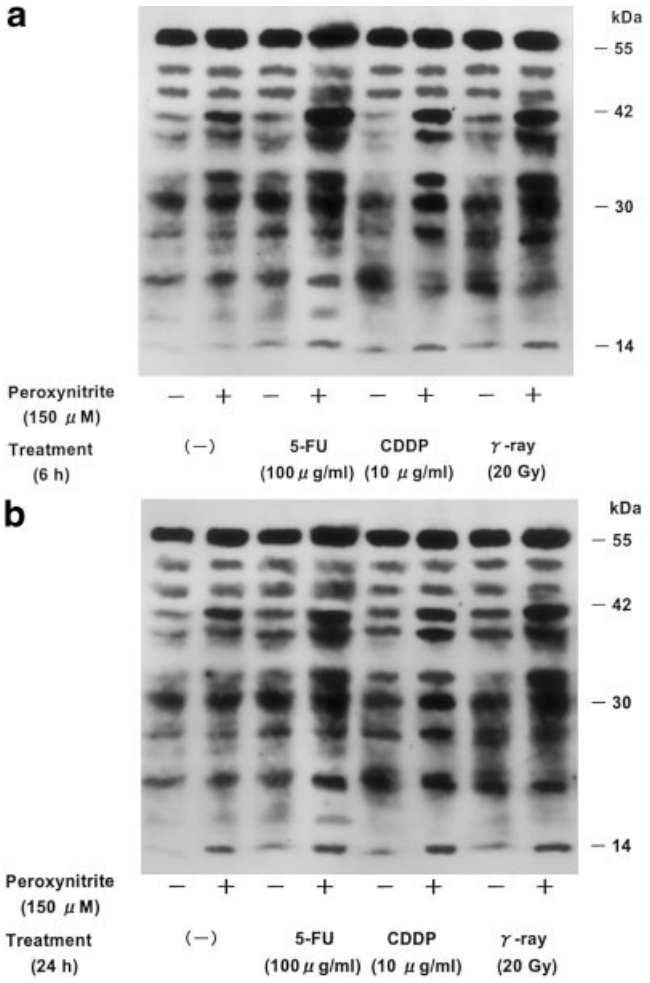

FIGURE 3 – The influence of $ONOO^-$ and anticancer agents on tyrosine nitration. OSC-4 cells were treated with each indicated dose of each agent in the presence or absence of peroxynitrite (150 μM) and they were cultivated for 6 hr (*a*) or 24 hr (*b*) at 37°C. After harvesting the cells, proteins were extracted, electrophoresed and blotted with anti-nitrotyrosine antibody.

Assay of cytochrome c activity

Cytochrome c from horse hearts (Sigma) (0.5 μg/ml) were treated with 0.1–1,000 μM peroxynitrite for 1 hr at 37°C. To assess whether the tyrosine-nitrated cytochrome c can induce an activation of Apaf-1 and caspase 3, an S100 fraction containing Apaf-1, procaspase-9 and procaspase 3 was prepared from HeLa cells as follows. HeLa cells (3×10^6) were suspended in 500 μl of buffer A (50 mM HEPES-KOH, pH 7.5 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol) containing 250 mM sucrose and a mixture of protease inhibitors (1 mM PMSF, 1% aprotinin, 1 mM leupeptin, 1 μg/ml pepstatin A and 1 μg/ml chymostatin). The cells were homogenized in the same buffer solution, with a glass Pyrex homogenizer using a Type B pestle (40 strokes). Unbroken cells, large plasma membrane fragments and nuclei were removed by centrifugation at 1,000*g* at 4°C for 10 min. The resulting supernatant was subjected to centrifugation at 10,000*g* at 4°C for 20 min. The supernatant was recentrifuged at 100,000*g* at 4°C for 1 hr and the final supernatant was used as the S100 fraction. Cytochrome c, which was treated with or without peroxynitrite, was mixed with the S100 fraction for 4 hr at 37°C. The mixture was used for the sample for Western blot analysis using anti-cleaved caspase-3 antibody (Cell Signaling Technology, Beverly, MA).

Western blot analysis and immunoprecipitation

Whole-cell extracts were prepared and Western blot analysis was carried out as described by An and Dou.[36] To perform a coupled immunoprecipitation-Western blot analysis, the whole cell extract (200 μg protein/ml) was incubated with protein A agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4°C for 2 hr. The collected supernatant was incubated for greater than 3 hr with 10 μl of agarose beads conjugated with either anti-cytochrome c antibody (Pharmingen, San Diego, CA) or anti-caspase 3 antibody (Transduction Laboratories, Lexington, KY), followed by incubation with Protein A beads at 4°C overnight. The washed immunoprecipitates were boiled in SDS sample buffer and used for Western blotting with antibody to 3-nitrotyrosine (UBI, Lake Placid, NY).

OSC-4 cells were pretreated with 150 μM $ONOO^-$ for 1 hr at 37°C and irradiated with 20 Gy of γ-rays or treated with 100 μg/ml of 5-FU, 10 μg/ml of CDDP or 10 μg/ml of PLM. After 48 hr from the start of treatment, cells were harvested. Cleavage of PARP and ICAD was then detected by Western blot analysis using anti-cleaved PARP antibody (Cell Signaling Technology) and anti-DFF45 antibody (ProSci Inc., Poway, CA).

Assay of caspase 3 activity

Recombinant procaspse-3 (MBL, Nagoya, Japan) (0.5 μg/ml) and recombinant active caspase 3 (MBL) (0.5 μg/ml) were treated with 0.1–1000 μM peroxynitrite or 25–400 μM SNP or SIN-1 for 1 hr at 37°C. Then, caspase 3 activity was detected using a caspase 3 colorimetric kit (Sigma), based on the hydrolysis of Ac-DEVD-*p*-nitroaniline (pNA) by caspase 3, resulting in the release of the pNA moiety. PNA was detected at 405 nm (ϵmM = 10.5). The specificity for caspase 3 activity was demonstrated by inhibition with 10 nM Ac-DEVD-CHO.

RESULTS

Intracellular NO and O_2^- levels after OSC-4 cell treatment with NO donors and anticancer agents

The intracellular NO level was dose-dependently increased by SNP and SIN-1 (Fig. 1). In the presence of 10 μM, 100 μM and 1,000 μM of SNP, intracellular NO was increased up to 132, 147.5 and 161 arbitrary units, respectively. A similar increase in the NO level was observed by the addition of SIN-1, although the degree of the increase was lower than that in SNP. The anticancer agents upregulated the intracellular NO level but the increases were weak. The maximal NO levels after treatment with 5-FU (100 μg/ml), PLM (10 μg/ml), CDDP (10 μg/ml) and γ-rays (20 Gy) were 114, 113, 112.5 and 106 arbitrary units, respectively.

Generation of O_2^- was enhanced by all anticancer agents examined (Fig. 2). Of the agents, 5-FU most strongly increased cytosolic and intramitochondrial O_2^- levels. The highest levels in both fractions were 181 ± 7 and 85 ± 6 arbitrary units, respectively, which were about 181.5% and 152.9% of each control level, respectively. Near increases of O_2^- levels was observed in CDDP and PLM-treated OSC-4 cells, but the increase of O_2^- by γ-rays was remarkably weaker than that by 5-FU.

Tyrosine nitration of cellular proteins and cytochrome c

Tyrosine nitration was weakly observed in 14, 25, 32, 40 and 42 kDa proteins from cell lysates of non-treated OSC-4 cells and the nitration levels in these proteins were weakly increased when OSC-4 cells were treated with 100 μg/ml 5-FU, 10 μg/ml CDDP for 6 hr or irradiated with ^{137}Cs as much as 20 Gy (Fig. 3*a*). The nitration of the tyrosine residues was further increased after the 24-hr treatment with the same dose of each above agent (Fig. 3*b*). The tyrosine nitration was furthermore strengthened by the addi-

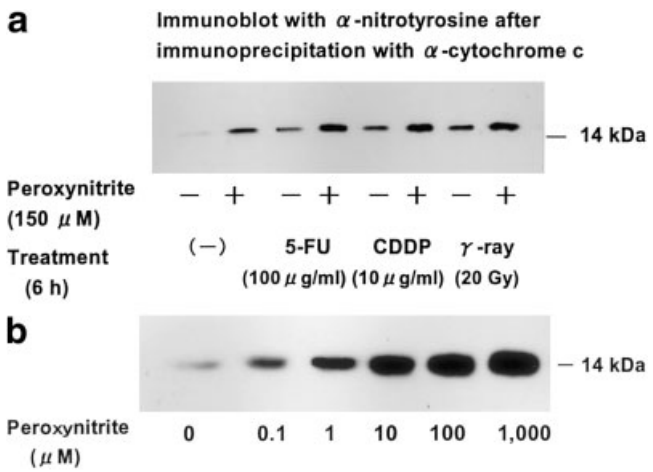

FIGURE 4 – The influence of $ONOO^-$ and anticancer agents on tyrosine nitration of cytochrome c. OSC-4 cells were treated with each indicated agent in the presence or absence of $ONOO^-$ (1 mM) for 6 hr at 37°C. Proteins extracted from the OSC-4 cells were then reacted with anti-cytochrome c antibody and the obtained immunoprecipitates were immunoblotted with anti-nitrotyrosine antibody (*a*). Purified cytochrome c extracted from horse hearts (0.5 μg/ml) was treated with each indicated dose of $ONOO^-$ for 1 hr at 37°C and nitrated tyrosine was immunoblotted with anti-nitrotyrosine antibody (*b*).

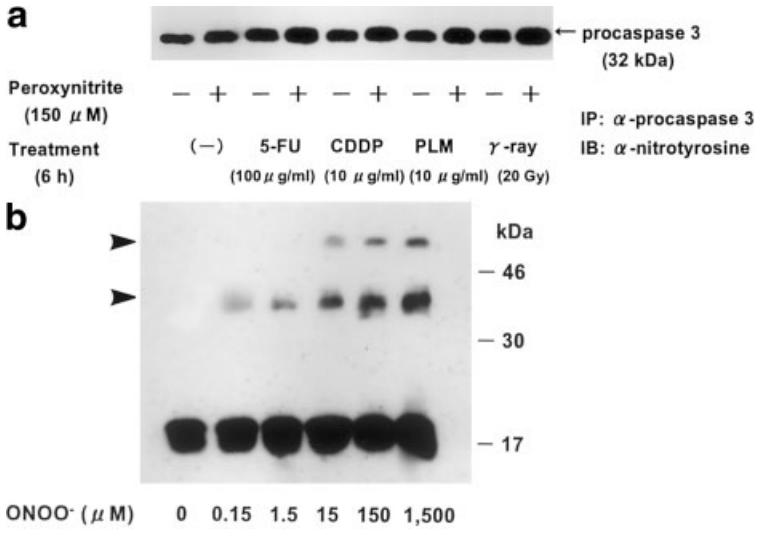

FIGURE 5 – Tyrosine nitration of procaspase 3 and cleaved caspase 3. OSC-4 cells were treated with each anticancer agent in the presence or absence and cultured for 6 hr at 37°C. Proteins were obtained from the cells and reacted with anti-procaspase 3 antibody and the immunoprecipitates were blotted with anti-nitrotyrosine antibody (*a*). Cleaved caspase 3 (0.5 μg/ml) was treated with 0.15 μM to 1,500 μM of $ONOO^-$ for 1 hr at 37°C and the cleaved caspase 3 was electrophoresed and immunoblotted with anti-cleaved caspase 3 antibody (*b*).

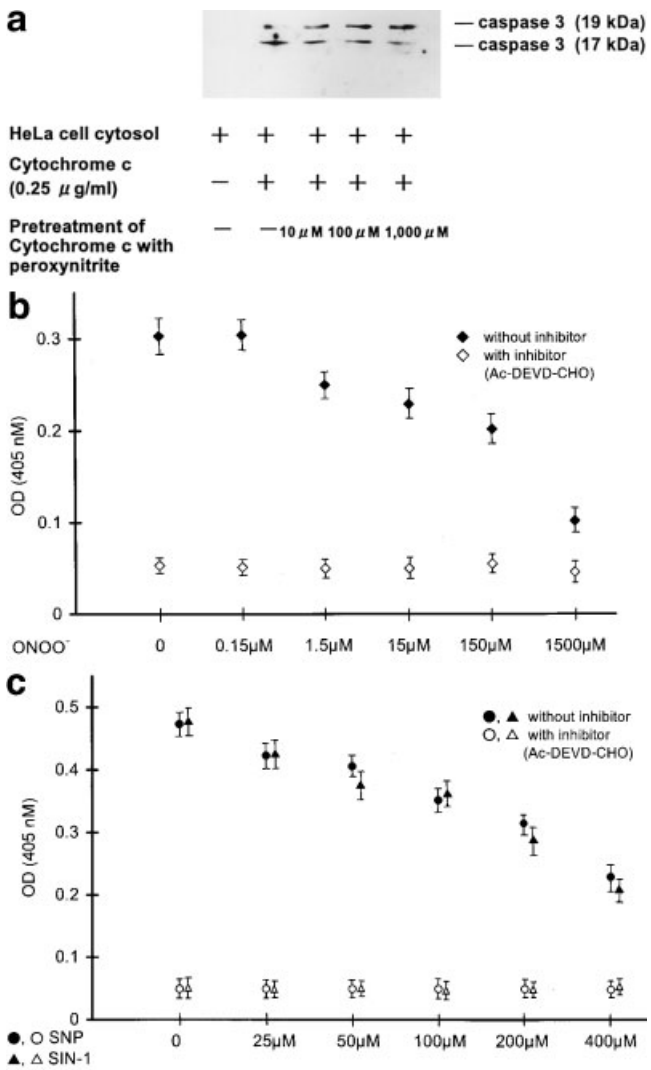

FIGURE 6 – Influence of tyrosine nitration on cytochrome c and cleaved caspase 3 activities. After treatment with $ONOO^-$ (10 μM to 1,000 μM) for 1 hr, cytochrome c (0.25 μg/ml) was added to the cytosol fraction obtained from HeLa cells and the mixture was incubated for 4 hr at 37°C. The reaction mixture was then electrophoresed and immunoblotted with anti-cleaved caspase 3 antibody (*a*). Cleaved caspase 3 (0.5 μg/ml) was treated with $ONOO^-$ (0.15–1500 μM) (*B*) or NO donors (SNP and SIN-1, 25–400 μM) (*c*) for 1 hr. The activity of the cleaved caspase 3 was then analyzed by absorbance at 405 nm of the pNA that was released from Ac-DEVD-pNA, the substrate of caspase 3. The specificity of the activity was ascertained by adding the inhibitor of Ac-DEVD-CHO.

tion of 150 μM $ONOO^-$ to the culture medium. The immunoblot with anti-nitrotyrosine antibody of precipitates by anti-cytochrome c antibody showed that nitration of tyrosine residues of cytochrome c was induced by treatment of OSC-4 cells with 5-FU, CDDP and γ-rays and the nitration levels were increased in the presence of peroxynitrite (Fig. 4*a*). The 14 kDa protein, purified cytochrome c from horse hearts, underwent nitration by $ONOO^-$ in a dose-dependent manner (Fig. 4*b*). Cytochrome c showed a weak nitration of the tyrosine-residues in the absence of $ONOO^-$ and the nitration reached the maximal level in the presence of 100 μM $ONOO^-$.

Tyrosine nitration of procaspase 3 and cleaved caspase 3

Enhanced tyrosine nitration of procaspase 3 by treatment with 5-FU, CDDP, PLM and γ-rays was observed by immunoblotting

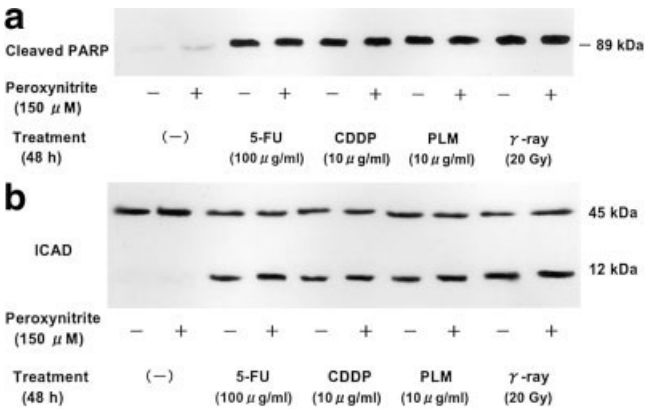

FIGURE 7 – Influence of $ONOO^-$ on the apoptosis induction. OSC-4 cells were pretreated with 150 μM $ONOO^-$ for 1 hr and treated with each anticancer agent. After 48 hr from the start of the second treatment, the cells were harvested and subjected to Western blot analysis for cleaved PARP (*a*) and ICAD (*b*).

of the caspase 3 immunoprecipitates lysates with anti-nitrotyrosine AB (Fig. 5*a*). The bands of tyrosine-nitrated procaspase 3 were thickened after 24 hr treatment with each anticancer drug and γ-rays although the increase in nitration by 3 hr treatment was very weak. Cleaved (active) caspase 3 was treated with $ONOO^-$ but nitration of the tyrosine residues was not observed (data not shown) and the cleaved caspase 3 was blotted at 34 kDa and 51 kDa (Fig. 5*b*).

Influence of tyrosine nitration on cytochrome c and cleaved caspase 3 activities

To examine the influence of tyrosine nitration of cytochrome c on its activity, cytochrome c was treated with 10 μM to 1,000 μM $ONOO^-$ for 1 hr and mixed with the cytosol obtained from HeLa cells. By mixing, procaspase 3 was cleaved into 17 and 19 kDa proteins (Fig. 6*a*). The cleavage levels in the addition of $ONOO^-$-pretreated cytochrome c was similar to that in the addition of $ONOO^-$-untreated cytochrome c.

The cleaved caspase 3 activity was not suppressed by 150 nM $ONOO^-$ and only slightly decreased by 1.5 μM, 15 μM and 150 μM $ONOO^-$ although the activity was strongly diminished by 1,500 μM $ONOO^-$ (Fig. 6*b*). NO donors, SNP and SIN-1, suppressed the caspase 3 activity in a dose-dependent manner but the suppression of caspase 3 activity by SNP and SIN-1 up to the concentration of 50 μM was very weak, although the activity was decreased down to about three-fifths and half of the control level by 200 μM and 400 μM of SNP and SIN-1, respectively (Fig. 6*c*).

Influence of $ONOO^-$ on the apoptosis induction

Pretreatment of OSC-4 cells with 150 μM $ONOO^-$ did not affect 5-FU-, CDDP-, PLM- and γ-ray-induced cleavage of PARP (Fig. 7*a*) and ICAD (Fig. 7*b*) and similar levels of cleavage of both proteins were observed in OSC-4 cells with and without the pretreatment. The DNA fragmentation induced by these anticancer agents was not decreased by 150 μM $ONOO^-$ and no influence of $ONOO^-$ on apoptosis of OSC-4-cells was ascertained by Nick-end labeling immunochemical stainings (data not shown).

DISCUSSION

There are some signal pathways involved in the induction of apoptosis. One of the pathways is the Fas (CD95/APO-1)-mediated signal flow and the other pathway is associated with the release of mitochondrial cytochrome c, which is under the regulation of Bcl-2 family proteins.[8–10] Although the cytochrome c release is induced by the signal from Fas, the main signal for the release of cytochrome c is the reduction of mitochondrial mem-

brane potential associated with ROS with an involvement of Bcl-2 family proteins.[35–41] In addition, ROS also play an important role in protein phosphorylation and activation of nuclear factor-kappa B (NF-κB) and activating protein-1 (AP-1), which are both associated with apoptosis.[42,43] In OSC-4 cells used in the present study, the main apoptotic pathway seemed to be associated with cytochrome c release from the mitochondria.[6]

ROS react with NO and highly reactive molecules are generated. Of these, $ONOO^-$, which is formed by the reaction of NO and O_2^-, is extremely reactive.[22,23] The impairment of proteins and DNA by $ONOO^-$ has been studied thoroughly.[15–19] In addition, $ONOO^-$ induces nitration of proteins and DNA. Many investigators have reported that nitration of tyrosine residues impaired the function of the protein by suppressing the phosphorylation of the protein and decreasing the enzyme activity.[30,44–50] It was reported recently that nitration of a c-Src kinase induced cell growth in an adenocarcinoma cell line and that tyrosine nitration had no effect on the function of some proteins associating with cell death.[51,52] These contradicting findings explain the unexploration of the influence of protein nitration.

In the present study, nitration of tyrosine residues of some proteins was observed in untreated cancer cells. This result seems to suggest that NO and ROS are generated *de novo* and nitric compounds are synthesized. The nitration level was, however, increased by cell treatment with γ-rays and anticancer drugs. The increase in protein nitration was coordinated with the 10–20% increase in the intracellular NO level and 40–80% increase in the O_2^- level. Although the estimation method of intracellular $ONOO^-$ has not been developed and we could not examine the $ONOO^-$ concentration in the cells, the results showed that $ONOO^-$ was generated intracellularly by the treatment with the anticancer agents and the generated $ONOO^-$-induced protein nitration.

An increased nitration of tyrosine residues of cytochrome c and procaspase 3 was shown by the Western blots of their immunoprecipitates and it was clarified that tyrosine nitration was increased by $ONOO^-$ in a dose-dependent manner. The protease activities of cytochrome c and caspase 3 were, however, scarcely decreased by $ONOO^-$ up to 150 μM although more higher concentrations of $ONOO^-$ largely decreased their activities. The intracellular NO levels in γ-rays and anticancer drug-treated cells were low and the $ONOO^-$ levels in the treated OSC-4 cells were suggested to be lower than 150 μM. The low NO levels suggest that in cancer chemoradiotherapy, any suppression of cytochrome c or caspase 3 activity by nitration is not induced even if some degrees of $ONOO^-$ are generated in the cancer cells. This seems to be supported by the finding that a relatively high concentration of $ONOO^-$ did not suppress the cleavage of ICAD and PARP by caspase 3.

Cassina *et al.*[28] reported that of 4 tyrosine residues of cytochrome c, Tyr-67 was nitrated by $ONOO^-$ and that the heme-vicinal Tyr-67 nitration promoted a conformational change in cytochrome c decreasing its activity. Our present study, however, showed no decrease in the cytochrome c activity by nitration when the $ONOO^-$concentration was lower than 150 μM. The lack of influence on the activity is probably derived from the finding in our present study that the nitrated tyrosine residue(s) was far from the domain that is responsible for the activity of cytochrome c.

Tyrosine nitration was observed in procaspase 3 but was not observed in cleaved, activated caspase 3. Procaspase 3 possesses 10 tyrosine residues and cleaved caspase 3 has 3 (Tyr-37, Tyr-41, Tyr-83). These 3 residues seemed not to be nitrated. The non-nitration of these tyrosine residues is probably associated with the non-decrease in cleaved caspase 3 activity in $ONOO^-$-treated cells.

When cleaved caspase 3 was treated with $ONOO^-$ and blotted with anti-cleaved caspase 3 antibody, bands of 34 kDa and 51 kDa were observed and the expression of these bands was increased in an $ONOO^-$ dose-dependent manner (Fig. 5*b*). These bands suggested dimerization and trimerization of the cleaved caspase 3. As described above, the cleaved caspase 3 activity was largely suppressed by $ONOO^-$ concentrations higher than 150 μM and the dimerization was strong at the high concentrations of $ONOO^-$. There is a possibility that severe polymerization of cleaved caspase 3 is responsible for the inactivation of cleaved caspase 3. To accurately decide the influence of protein nitration, the relation between the position of nitrated tyrosines and caspase 3 activity should be determined.

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