Original Article

Protective effects of resveratrol against 5-fluorouracil-induced oxidative stress and inflammatory responses in human keratinocytes

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Although 5-fluorouracil (5-FU) is currently used as an anti-cancer chemotherapy, adverse effects such as oral mucositis potentially limit its clinical application. Additionally, the prevention of 5-FU-induced side effects are scarce. Resveratrol is known to decrease oxidative damage and inflammation. In this study, we examined the protective effects of resveratrol on 5-FU-induced oxidative stress and inflammatory responses in normal human keratinocytes (HaCaT cell) as *in vitro* oral mucositis model. HaCaT cells were exposed to 5-FU and simultaneously treated with resveratrol. The effects of resveratrol on 5-FU-induced cytotoxicity were evaluated using cell viability assay. The production of reactive oxygen species (ROS) was measured using a fluorescence spectrophotometer. The effects of resveratrol on nuclear factor erythroid 2-related factor 2 (Nrf2), silent information regulator transcript-1 (SIRT-1), and nuclear factor kappa B (NF-κB) signaling and inflammatory cytokine expression were examined. Resveratrol suppressed 5-FU-induced overproduction of ROS by upregulating anti-oxidant defense genes through Nrf2 activation and SIRT-1 expression. Concerning inflammatory responses, resveratrol suppressed the 5-FU-induced expression of pro-inflammatory cytokines via NF-κB nuclear translocation. Conversely, *N*-acetylcysteine reduced ROS levels without affecting the expression of pro-inflammatory cytokines. Resveratrol might be useful for preventing 5-FU-induced adverse effects by activating anti-oxidant and anti-inflammatory responses.

***Key Words*: oxidative stress, inflammation, oral mucositis, resveratrol, human normal keratinocyte**

The pyrimidine anti-metabolite 5-fluorouracil (5-FU) is widely used to treat a variety of cancers; however, it causes several side effects. Oral mucositis, a painful inflammatory ulcer of the oral mucosa, is a common side effect occurring in up to 90% in patients with cancer treated with 5-FU.[1,2] The clinical manifestation of oral mucositis is ulceration of the mucosal epithelium. In addition, the formation of ulcers causes severe pain, altered taste, dry mouth, and decreased appetite, and it directly or indirectly induces the suppression of nutritional uptake in many cases.[3–5] Despite the availability of palliative treatments including antibiotics, analgesics, and radioprotective agents, in addition to general oral hygiene modification, these symptomatic therapies have limited efficacy.[6]

Recent studies illustrated that the pathogenesis of oral mucositis is based on reactive oxygen species (ROS) generation and inflammation.[2,3] The overproduction of ROS leads to their accumulation and oxidative stress, which further aggravate cell damage and eventually cause apoptosis.[7] Therefore, cells scavenge the overproduced ROS through anti-oxidant networks, and the balance between ROS production and scavenging is tightly regulated.[8] Nuclear factor erythroid 2-related factor 2 (Nrf2) is a pleiotropic protein and important anti-oxidant sensor. Under oxidative stress, electrophiles and oxidants modify Kelch-like ECH-associated protein-1, leading to its dissociation from Nrf2. Following its release, Nrf2 translocates to the nucleus and then binds to an anti-oxidant response element (ARE) sequence to mediate the transcription of target genes, such as heme oxygenase 1 (HO-1) and NAD(P)H-quinone oxidoreductase (NQO-1).[9] Transcription of these genes increases the resistance of cells to oxidative stress and protects against inflammation.[10]

Resveratrol is a polyphenolic phytoalexin that naturally occurs in many plant products, such as grapes, berries, and peanuts.[11] It has been reported that resveratrol is more effective than conventional anti-oxidants such as *N*-acetylcysteine (NAC) and quercetin in decreasing oxidative damage.[12] The cellular effects of resveratrol were attributed to its anti-inflammatory and anti-oxidant capacities. However, the protective effects of resveratrol on 5-FU-induced damage in normal keratinocytes remain to be determined. Furthermore, resveratrol has been reported to enhance 5-FU-induced cytotoxicity in cancer cells.[13]

In this study, a normal human keratinocyte cell line (HaCaT) was used as an *in vitro* model to investigate oral mucositis caused by 5-FU, as these cells can serve as a useful model for oral mucositis research.[14] Several studies suggested that HaCaT cells can produce pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6,[15] and tumor necrosis factor (TNF)-α.[16] However, the roles of ROS production and upregulation of the nuclear factor kappa B (NF-κB) pathway induced by 5-FU in normal keratinocytes have not been explored.

We hypothesized that resveratrol can prevent or attenuate oral mucositis caused by 5-FU. We evaluated the inhibitory effects of resveratrol on 5-FU-induced oxidative stress and inflammatory responses. Moreover, we investigated the effects of resveratrol on the Nrf2, silent information regulator transcript-1 (SIRT-1), and NF-κB signaling pathways.

Materials and Methods

Cell culture. The HaCaT immortalized keratinocyte cell line from adult human skin was obtained from German Cancer Research Center (Heidelberg, Germany). Cells were plated in cell culture flasks and suspended in Dulbecco's Modified Eagel

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Table 1. Primers used in this study for real-time PCR

Gene Name	Forward Primer	Reverse Primer
IL-1β	TGATGGCTTATTACAGTGGC	TGTAGTGGTGGTCGGAGATT
IL-6	AAGCCAGAGCTGTGCAGATGAGTA	TGTCCTGCAGCCACTGGTTC
TNF-α	CCTCTGGCCCAGGCAGTCAGA	GGCGTTTGGGAAGGTTGGAT
IL-10	GCTGCACCCACTTCCCAGGC	CCTGCTCCACGGCCTTGCTC
HO-1	CTTTCAGAAGGGTCAGGTGTC	TGCTTCTTTCGCTCTATCTCC
NQO-1	CATCATTTGGGTCAAGTCC	ACAGCCGTGGCAGAACTA
SIRT-1	AAGGAAAACTACTTCGCAAC	GGAACCATGACACTGAATTA
GAPDH	GTATTGGGCGCCTGGTCACC	CGCTCCTGGAAGAGATGGTGATGG

Medium (DMEM; Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum (MP Biomedicals LLC, Santa Ana, CA), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Sigma-Aldrich, St. Louis, MO), and kept in a humidified incubator at 37°C, with an atmosphere of 5% CO_2.

Cell viability. The cell viability assay was conducted to determine the concentrations of resveratrol as a chemopreventive agent against toxicity induced by 5-FU. Briefly, HaCaT cells were seeded into the 96-well plate and incubated for 24 h. Then cells treated with or without different concentrations of resveratrol and 5-FU for 24 h. WST-1 (Takara Bio Inc., Otsu, Japan) assay was performed according to the standard protocol of manufacture. Absorbance value was measured at 450 nm by a microplate reader. Cell viability was expressed as the percentage of untreated cells (control).

Production and detection of ROS. HaCaT cells were incubated with 5-FU in presence or absence of resveratrol for 24 and 48 h. The adherent cells were stained with CellROX Green Reagent (Life Technology, Carlsbad, CA) at 37°C in dark for 30 min, nuclei were stained by 4',6-diamidino-2-phenylindole (DAPI) for 15 min, and harvested in PBS. The cell suspension (10,000 cells/well) was placed in a flat bottom 96-well assay plate (Nunc/ThermoFisher scientific, Waltham, MA). The intensity was then immediately measured using the fluorescence spectrophotometer (Tecan M200 Infinite PRO, Tecan Japan Co, Kawasaki, Japan) at an excitation wavelength 485 nm and emission wavelength 520 nm.

Immunofluorescence staining. HaCaT cells were incubated and then treated with 5-FU in the presence or absence of resveratrol for 24 h. Subsequently, the cells were fixed with 4% paraformaldehyde for 15 min followed by permeabilized with 0.5% Triton X-100. The cells were blocked with Blocking One Histo (Nacalai Tesque) for 10 min to prevent non-specific labelling. The cells were incubated with a monoclonal antibody specific to Nrf2 (sc-722, Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C, then incubated with a secondary antibody, anti-rabbit IgG Fab2 Alexa Fluor 488 conjugate (Cell Signaling Technology, Inc., Beverly, MA) for 1 h. Nuclei were stained with DAPI for 15 min. Images were captured using a fluorescent microscopy BZ-X800 (Keyence Co., Osaka, Japan) at 400× magnification.

Extraction of protein and Western blot analysis. The nuclear protein extracts were prepared from HaCaT cells using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Rockford, IL), according to the manufacturer's protocol. Additionally, total protein was extracted by ice-cold RIPA buffer (Nacalai Tesque) with protease inhibitor cocktail. The protein samples were subsequently prepared 4× Laemmli Sample Buffer (Bio-Rad Laboratories, Forester City, CA) and boiled at 100°C for 5 min. Equal amounts of protein were separated by SDS-PAGE and then transferred onto polyvinylidene difluoride membranes for 35 min at 100 V. After that, the membranes were blocked with Blocking One (Nacalai Tesque) for 40 min at room temperature. The membranes were incubated at 4°C overnight in each primary antibody. Primary antibodies specific to SIRT-1, HO-1, NF-κB, histone H3 and β-actin (Cell Signaling Technology) and Nrf2 were used. Membranes were washed by TBST three times and incubated with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology). To detect the immunocomplex, an Immobilon HRP substrate (Bio-Rad Laboratories) was used. Immunoblots were scanned by densitometry and the intensity was quantified by using Image Lab software (Bio-Rad Laboratories). The relative levels of SIRT-1 and HO-1 were normalized to β-actin, and nuclear Nrf2 and NF-κB were standardized to histone H3.

Real-time PCR. HaCaT cells were sub-cultured and treated with 5-FU in the presence or absence of resveratrol and NAC (Sigma-Aldrich) for 24 and 48 h. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was reverse transcribed using ReverTra Ace (Toyobo, Osaka, Japan). Real-time PCR was performed using SYBR Green (Bio-Rad Laboratories) and a real-time PCR system (Bio-Rad Laboratories) according to the following cycling parameters: initial denaturation at 95°C for 1 min, 40 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 30 s and extension at 72°C for 25 s. The primer sequences were showed in Table 1. The mRNA levels were calculated by determining the relative expression compared with GAPDH and the Ct of the control was normalized to 1.

ELISA assay. HaCaT cells were seeded and preincubated for 24 h. For the next 48 h, the cells treated with 5-FU in the presence or absence of resveratrol. The culture supernatants were collected from both of treated and untreated cells, and centrifuged to remove cell debris. We used commercially available ELISA kits (Biolegend, San Diego, CA), according to the manufacturer's instructions. The concentrations of cytokines were calculated according to standard curves, and the obtained results are shown as the amount of IL-1β, IL-6, and TNF-α (pg/ml).

Statistical analysis. For statistical analysis, differences between the groups were analyzed with one-way analysis of variance (ANOVA) and Tukey's post hoc test for multiple comparisons using JMP (ver. 12, SAS Institute, Cary, NC). A value of $p<0.05$ was regarded as statistically significant.

Results

Effects of resveratrol on 5-FU-induced cytotoxicity in HaCaT cells. The cytotoxicity of resveratrol and 5-FU (5 μg/ml) in HaCaT cells was evaluated after 24 h of treatment. We first examined the effects of various concentrations of resveratrol (0.1–1,000 μM) on the viability of HaCaT cells in the presence and absence of 5-FU. Higher concentrations of resveratrol (>100 μM) reduced HaCaT cell viability (Fig. 1A), whereas lower concentrations (≤100 μM) had no influence on

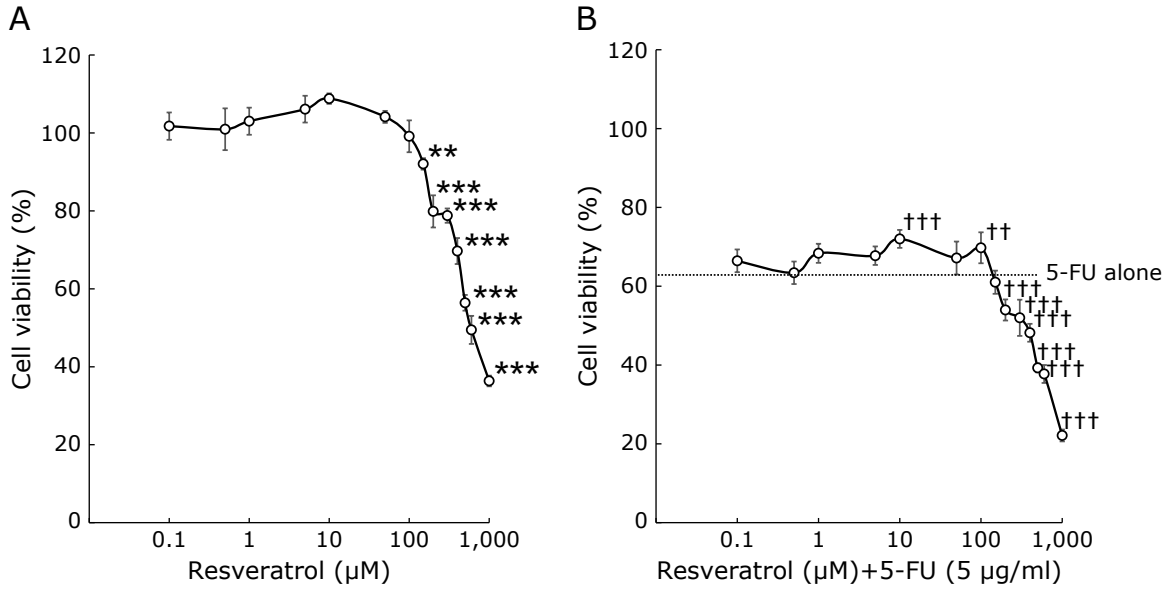

Fig. 1. Effects of resveratrol on 5-FU-induced cytotoxicity in HaCaT cells. HaCaT cells were treated with different concentrations of resveratrol alone (A) or 5-FU (5 μg/ml) and resveratrol (B) for 24 h, and then cell viabilities were evaluated by WST-1 assay. The cell viabilities were expressed as % of control. Results are presented as the means ± SD ($n = 6$). $^{**}p<0.01$, $^{***}p<0.001$ significantly different from control. $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ significantly different from 5-FU alone.

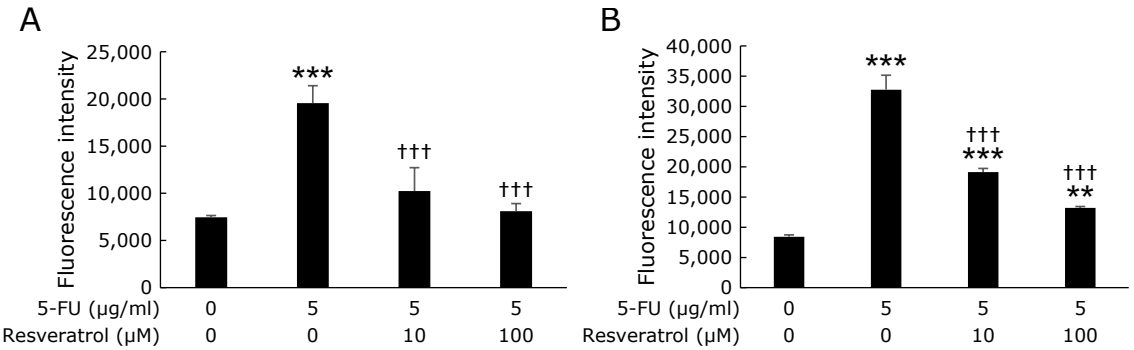

Fig. 2. Effects of resveratrol on the ROS production in HaCaT cells treated with 5-FU. HaCaT cells were incubated with 5-FU in the presence or absence of resveratrol for 24 h (A) and 48 h (B). Results are presented as the means ± SD of three independent experiments. $^{**}p<0.01$, $^{***}p<0.001$ significantly different from control. $^{\dagger\dagger\dagger}p<0.001$ significantly different from 5-FU alone.

viability. In addition, 5-FU (5 μg/ml) treatment alone for 24 h significantly decreased cell viability relative to the control (62.7 ± 0.3%). Although not all the lower concentrations of resveratrol had significantly increased the viability compared with 5-FU alone, there was a tendency for lower concentrations (1–100 μM) to protect cytotoxicity induced by 5-FU (Fig. 1B).

Resveratrol suppresses 5-FU-induced ROS generation by activating the Nrf2 pathway in HaCaT cells. To investigate the anti-oxidant effects of resveratrol, we measured ROS production in HaCaT cells. 5-FU treatment for 24 or 48 h resulted in significantly increased ROS production (Fig. 2). Additionally, resveratrol significantly decreased ROS production induced by 5-FU treatment (Fig. 2).

We examined the effects of resveratrol on the Nrf2/anti-oxidant pathway. Nuclear expression of Nrf2 was slightly observed in 5-FU-treated HaCaT cells, but resveratrol increased nuclear Nrf2 expression as observed via immunofluorescence and western blot analyses using nuclear extracts (Fig. 3A and B). Thus, resveratrol accelerated Nrf2 nuclear translocation in HaCaT cells. Additionally, the expression of the anti-oxidant enzymes NQO-1 and HO-1 were analyzed by real-time PCR. 5-FU and resveratrol increased the expression of NQO-1 and HO-1 compared to their control levels (Fig. 3D and F). We also found that the protein expression of HO-1 was significantly increased by resveratrol treatment compared to the effects of 5-FU alone (Fig. 3C). Moreover, resveratrol alone also increased the expression of NQO-1 and HO-1 compared to control levels (Fig. 3E and G). These findings indicate that resveratrol activates the Nrf2 pathway and upregulates anti-oxidant defense genes.

Resveratrol increases the levels of SIRT-1 in HaCaT cells. Resveratrol is known as a SIRT-1 activator. Indeed, the mRNA expression of SIRT-1 was increased by resveratrol compared to control level in HaCaT cells (Fig. 4A). Additionally, we examined SIRT-1 expression after 5-FU treatment with or without resveratrol using real-time PCR and western blotting. 5-FU treatment increased the mRNA expression of SIRT-1 in HaCaT cells (Fig. 4B). Moreover, resveratrol synergistically enhanced both the protein and mRNA levels of SIRT-1 compared

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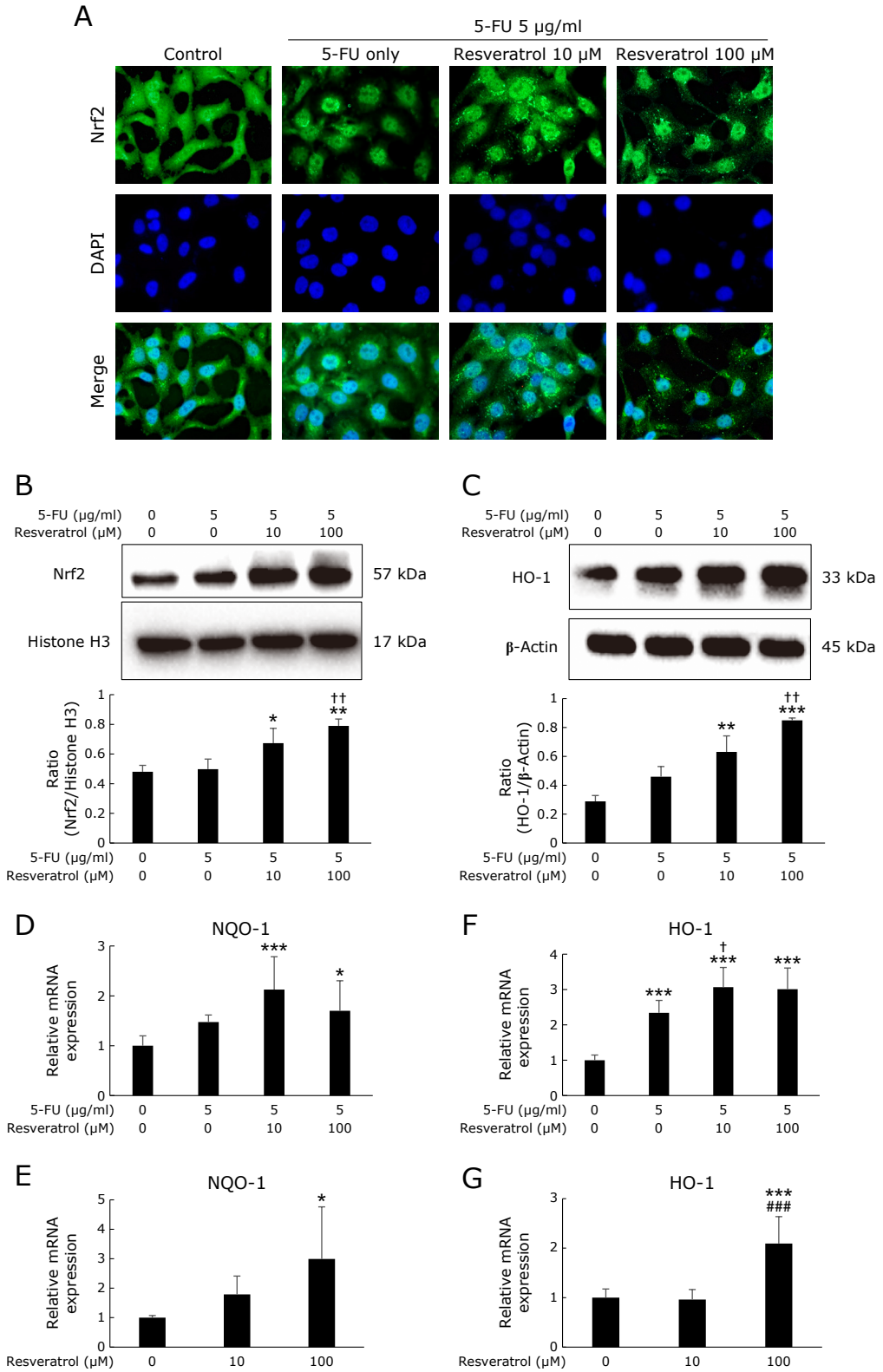

Fig. 3. Effects of resveratrol on the Nrf2 nuclear translocation, and expressions of HO-1 and NQO-1 in HaCaT cells treated with 5-FU. HaCaT cells incubated with 5-FU in the absence or presence of resveratrol for 24 h. (A) Immunofluorescence staining of Nrf2 for each treatment. Nuclei were labeled with DAPI. 400× magnification. (B, C) Representative blots and quantitative analyses are shown for nuclear Nrf2, histone H3, HO-1, and β-actin. (D–G) Relative mRNA levels of NQO-1 and HO-1 were determined by real-time PCR. Bars represent mRNA expression normalized to GAPDH and relative to the control. Results are presented as the means ± SD ($n = 6$). *p<0.05, **p<0.01, ***p<0.001 significantly different from control. $^{\dagger}p$<0.05, $^{\dagger\dagger}p$<0.01 significantly different from 5-FU alone. $^{\#\#\#}p$<0.001 significantly different from resveratrol (10 μM) alone.

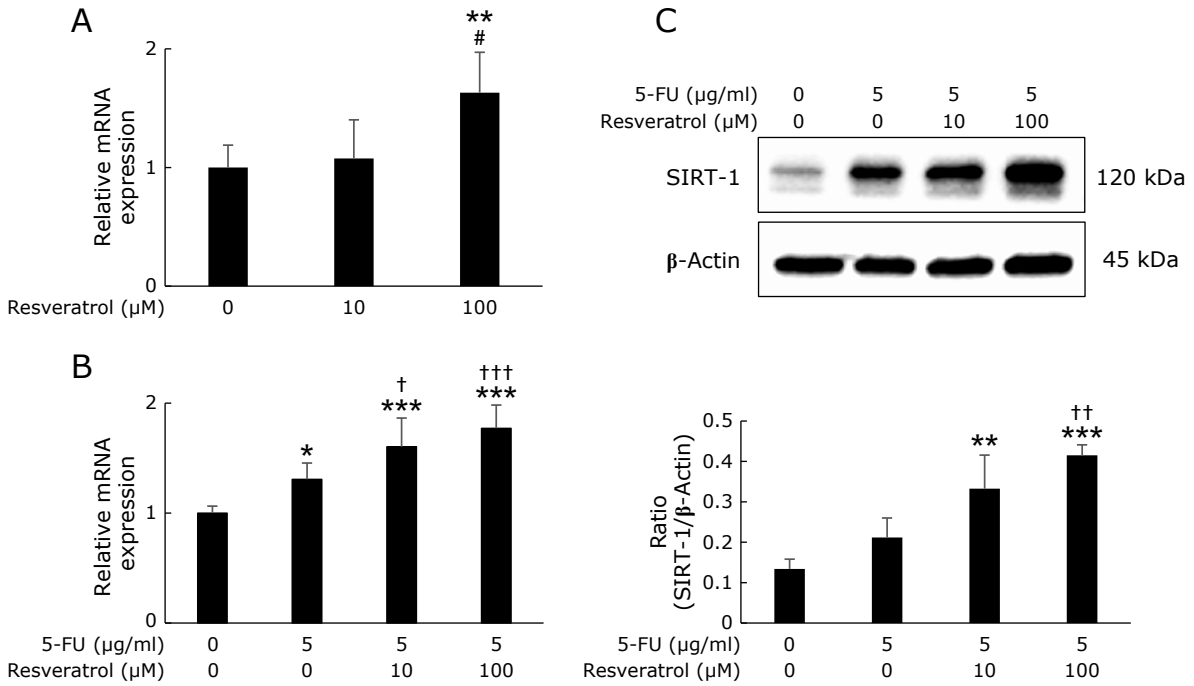

Fig. 4. Effects of resveratrol on the levels of SIRT-1 in HaCaT cells treated with 5-FU. HaCaT cells incubated with 5-FU in the absence or presence of resveratrol for 24 h. (A, B) Relative mRNA levels of SIRT-1 were determined by real-time PCR. Bars represent mRNA expression normalized to GAPDH and relative to the control. (C) Representative blots are shown for SIRT-1 and β-actin. Quantitative analyses are shown for SIRT-1/β-actin. *p<0.05, **p<0.01, ***p<0.001 significantly different from control. $^{\dagger}p$<0.05, $^{\dagger\dagger}p$<0.01, $^{\dagger\dagger\dagger}p$<0.001 significantly different from 5-FU alone. $^{\#}p$<0.05 significantly different from resveratrol (10 μM) alone.

to the effects of 5-FU alone (Fig. 4B and C).

Resveratrol inhibits 5-FU-induced NF-κB p65 nuclear translocation in HaCaT cells. The effect of resveratrol on the 5-FU-induced nuclear translocation of the NF-κB p65 subunit was examined (Fig. 5A). NF-κB p65 expression in the nucleus was significantly increased by 5-FU treatment, and resveratrol reduced the 5-FU-induced nuclear expression of p65. It is thus suggested that resveratrol inhibits the NF-κB pathway, which is activated by 5-FU. We also examined the effect of resveratrol alone on NF-κB expression. Resveratrol treatment had no significant influence on its expression (Fig. 5B).

Resveratrol suppresses 5-FU-induced inflammatory cytokine expression in HaCaT cells. The mRNA and protein levels of inflammatory cytokines in HaCaT cells were measured by real-time PCR (Fig. 6A–H) and ELISA (Fig. 6I and J), respectively. Significantly higher expression of IL-1β, IL-6, and TNF-α was observed after exposure to 5-FU at different time points (24 and 48 h; Fig. 6A–C and 6E–G, respectively). Resveratrol significantly decreased the expression of IL-1β, IL-6, and TNF-α induced by 48 h of 5-FU treatment (Fig. 6E–G). Further, the protein levels of IL-1β and IL-6, which were increased by 5-FU treatment, were also reduced after 48 h of resveratrol treatment (Fig. 6I–J). TNF-α protein levels were under the detection limit of ELISA in all conditions. We also investigated the effect of resveratrol on IL-10 expression, finding that the treatment had no significant influence on its expression (Fig. 6D, H, and K).

NAC suppresses 5-FU-induced ROS production but not pro-inflammatory cytokine expression. We examined whether NAC could decrease the expression of pro-inflammatory cytokines by suppressing the overproduction of ROS. As expected, NAC significantly decreased ROS production induced by 5-FU treatment (Fig. 7A). However, NAC did not reduce the expression of inflammatory cytokines induced by 5-FU treatment at 24 (Fig. 7B–D) and 48 h (Fig. 7E–G). This finding suggests that the expression of inflammatory cytokines in HaCaT cells following 5-FU treatment is not a downstream event of ROS production and is independent of oxidative stress.

Discussion

In this study, we explored the utility of resveratrol for treating chemotherapy-induced oral mucositis using an *in vitro* cell culture model. To the best of our knowledge, this was the first study to demonstrate the protective effects of resveratrol on oxidative stress and inflammation induced by chemotherapy in normal human keratinocytes. Additionally, our findings suggest that resveratrol exerts substantial anti-oxidant and anti-inflammatory effects by activating Nrf2 signaling and inhibiting NF-κB signaling, respectively.

5-FU has excellent effects against a variety of cancers including breast, head and neck, stomach, and skin cancers.[2] 5-FU blocks DNA replication and induces the overproduction of ROS and upregulation of inflammatory responses, thereby directly inducing toxicity in the oral mucosa.[17–19] 5-FU commonly causes oral mucositis in patients with cancer.[1] The pathogenesis of oral mucositis is divided into five biological steps: (i) initiation, (ii) primary damage response, (iii) signal amplification, (iv) ulceration, and (v) healing.[4] During the initial stage of oral mucositis, anti-cancer drugs induce the overproduction of ROS, which play a primary role in the activation of a variety of transcription factors, such as NF-κB.[20] Activation of NF-κB subsequently leads to the production of the inflammatory cytokines IL-1β, IL-6, and TNF-α.[19] The inflammatory cytokines interact synergistically with NF-κB, thereby amplifying the initial signals toward cell damage and tissue destruction, which occur in the primary damage response of oral mucositis.[20,21] We revealed that the nuclear translocation of NF-κB p65 (Fig. 5) and production of pro-inflammatory cytokines (Fig. 6) were significantly induced by 5-FU treatment in HaCaT cells, suggesting that the nuclear translocation of NF-κB p65

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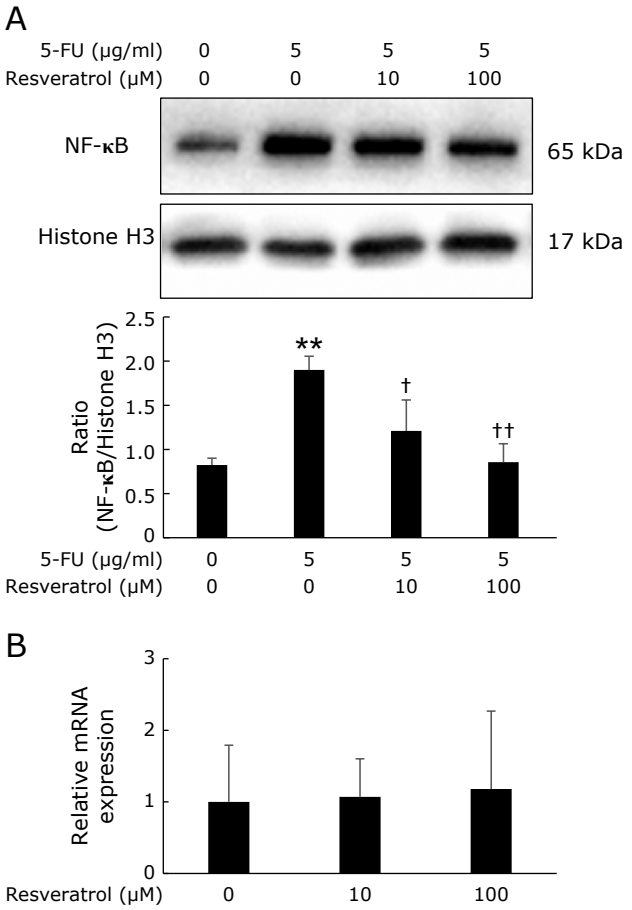

Fig. 5. Effects of resveratrol on nuclear translocation of NF-κB in HaCaT cells treated with 5-FU. (A) HaCaT cells were pre-treated with resveratrol for 3 h and then added 5 μg/ml of 5-FU for 6 h. Representative Western blots and quantitative analysis were shown. (B) Relative mRNA levels of NF-κB were determined by real-time PCR. Bars represent mRNA expression normalized to GAPDH and relative to the control. $^{**}p<0.01$ significantly different from control. $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ significantly different from 5-FU alone.

leads to the upregulation of pro-inflammatory cytokines.

5-FU treatment increased the levels of ROS in HaCaT cells (Fig. 2). There is a balance between the production and elimination of ROS in the healthy condition. However, chronic inflammation leads to sustained ROS overproduction. Therefore, it is believed that the uptake of exogenous anti-oxidants is beneficial for eliminating ROS and controlling oxidative stress in the body.[22,23] Previous researches have illustrated that resveratrol is the efficient hydroxyl radical and superoxide radical scavenger, which is substantial but less effective than known radical scavengers such as glutathione and ascorbic acid.[22,24] In addition, resveratrol has been reported to increase the levels of anti-oxidant genes, i.e., Nrf2, HO-1, and SIRT-1, in normal cells.[25,26] It has been also reported that resveratrol can regulate the expression and activity of anti-oxidant enzymes via the activation of the transcription factor Nrf2.[27] Our findings demonstrated that resveratrol could reduce the overproduction of ROS induced by 5-FU in HaCaT cells (Fig. 2). Moreover, resveratrol can promote the activity of SIRT-1, which regulates the activity of multiple downstream targets including the NF-κB pathway.[28,29] Consistent with these previous studies, resveratrol could controll oxidative stress with activating of Nrf2/anti-oxidant and SIRT-1 defense pathways as well as ROS scavenging.

Nrf2 is considered a main regulator of anti-oxidant defenses in mammals.[30] In response to oxidative stress, Nrf2 is activated, and it translocates to the nucleus, in which it binds to AREs and upregulates the expression of phase 2 enzymes, such as HO-1 and NQO-1, thereby maintaining the redox balance of cells.[31,32] We found that the nuclear translocation of Nrf2 was more strongly increased in HaCaT cells treated with both 5-FU and resveratrol than in cells treated with 5-FU alone (Fig. 3). In addition, the upregulation of HO-1 and NQO-1 expression was significantly induced by resveratrol (Fig. 3). As 5-FU-induced ROS overproduction was significantly suppressed by resveratrol (Fig. 2), the compound might regulate oxidative stress by activating the Nrf2/anti-oxidant pathway in normal cells.

SIRT-1 is a nicotinamide adenine dinucleotide-dependent nuclear histone deacetylase that participates in the regulation of several processes, such as inflammation, histone deacetylation, and apoptosis.[33–35] SIRT-1 reduces the levels of oxidative stress and the severity of inflammation.[36,37] Moreover, SIRT-1 promotes the nuclear accumulation, DNA binding, and transcriptional activities of Nrf2 by upregulating its downstream genes HO-1 and NQO-1.[38] In addition, the inhibition of Nrf2 ubiquitination by SIRT-1, which increases the stability and activity of Nrf2, suppresses apoptosis and resistance to oxidative stress.[39] In this study, we illustrated that resveratrol enhanced both SIRT-1 and nuclear Nrf2 levels in HaCaT cells (Fig. 3 and 4), suggesting that SIRT-1 promotes the stability and activity of Nrf2 under treatment with resveratrol. SIRT-1 also inhibits the transactivation of NF-κB.[35] In addition, HO-1 has a central role in NF-κB inhibition in the cytoplasm.[40] Therefore, we speculate that resveratrol can suppress the inflammatory responses activated by NF-κB through upregulating SIRT-1 and HO-1.

NF-κB activation is a cellular injury response that leads to inflammation via the production of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α.[41] In an inactive state, NF-κB exists in the cytoplasm as a heterodimer of p65 and p50 subunits, but once activated by the phosphorylation and degradation of inhibitor of kappa B, it translocates to the nucleus and activates the transcription of various pro-inflammatory genes.[42] Resveratrol does not affect the DNA-binding activity of NF-κB, but it inhibits the nuclear translocation of p65.[43] In the present study, differing from resveratrol, NAC significantly decreased the overproduction of ROS induced by 5-FU, and it did not affect the expression of inflammatory cytokines (Fig. 7). This suggests that 5-FU directly induces the upregulation of inflammatory cytokines rather than through the overproduction of ROS. Importantly, resveratrol might protect cells against the cytotoxicity of 5-FU via two independent pathways, i.e., anti-oxidant and anti-inflammatory signaling. In the latter, suppression of the 5-FU-induced nuclear translocation of NF-κB p65 is likely responsible for the downregulation of inflammatory cytokines.

In conclusion, resveratrol displayed multiple beneficial effects in an *in vitro* oral mucositis model induced by 5-FU. Resveratrol decreased 5-FU-induced ROS production by activating the Nrf2 and SIRT-1 pathways in normal human keratinocytes. Moreover, resveratrol suppressed inflammatory responses by reducing the production of inflammatory cytokines via the suppression of NF-κB signaling. Overall, our results support the hypothesis that resveratrol is a safe and beneficial candidate for preventing oral mucositis associated with cancer chemotherapy. However, further studies are required before the practical use of resveratrol supplements to prevent oral mucositis.

Author Contributions

SC contributed to perform all experiments and drafted the manuscript. NT contributed to design the study, perform experiments, and revise manuscript. YK contributed to perform immunofluorescence staining, and revise manuscript. TT and KM contributed to perform Western blot analysis. NI and HI

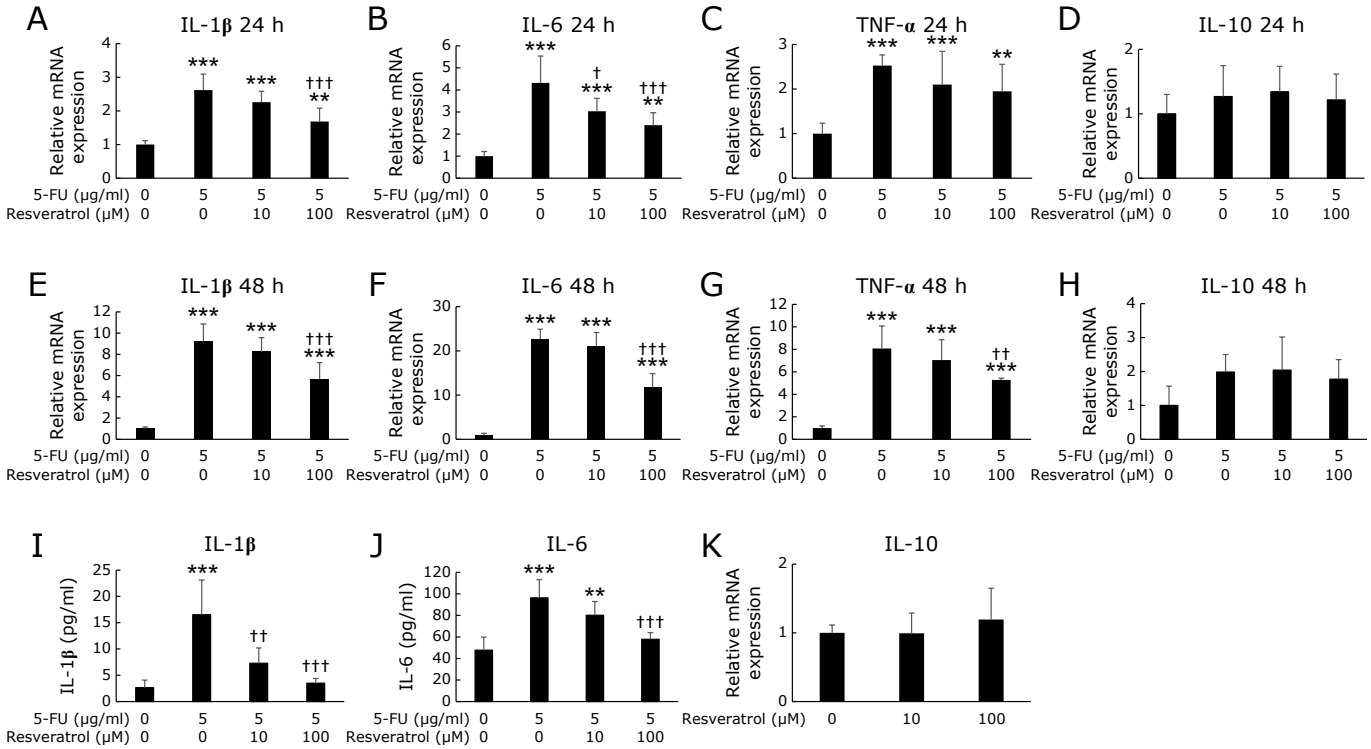

Fig. 6. Effects of resveratrol on 5-FU induced-inflammatory cytokines in HaCaT cells treated with 5-FU. HaCaT cells were treated with 5-FU in the presence or absence of resveratrol for 24 h (A–D, K) and 48 h (E–H). IL-1β, IL-6, TNF-α, and IL-10 levels were determined by real-time PCR. Bars represent mRNA expression normalized to GAPDH and relative to control. (I) IL-1β and (J) IL-6 amounts of culture supernatants of HaCaT cells at 48 h were determined by ELISA. Data are expressed as means ± SD ($n = 6$). $^{**}p<0.01$, $^{***}p<0.001$ significantly different from control. $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ significantly different from 5-FU alone.

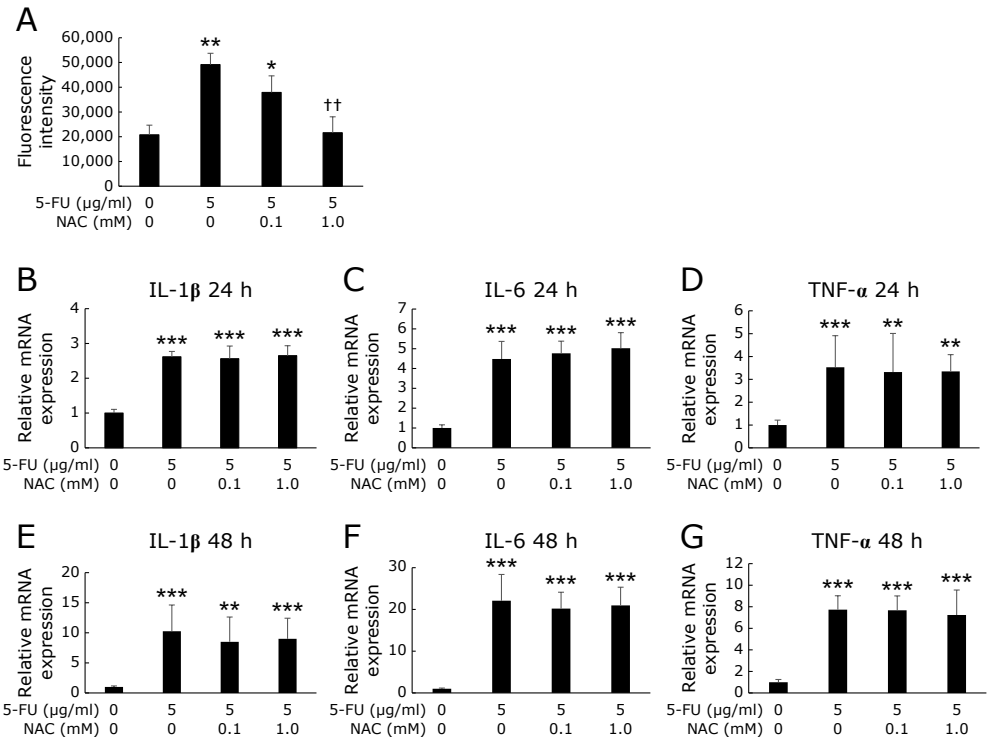

Fig. 7. Effects of NAC on the levels of production of ROS and pro-inflammatory cytokines in 5-FU treated HaCaT cells. HaCaT cells was incubated with 5-FU in the presence or absence of NAC for 24 h (A–D) and 48 h (E–G). (A) ROS assay. Results are presented as the means ± SD of three independent experiments. (B–G) IL-1β, IL-6, and TNF-α levels were determined by real-time PCR. Bars represent mRNA expression normalized to GAPDH and relative to control. Data are expressed as means ± SD ($n = 6$). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, significantly different from control. $^{\dagger\dagger}p<0.01$ significantly different from 5-FU alone.

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contributed to revise manuscript. All the authors read and approved the final manuscript.

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Abbreviations

ARE	antioxidant response element
5-FU	5-fluorouracil
HaCaT cell	immortalized human keratinocyte cell line
HO-1	heme oxygenase 1
IL-1β	interleukin (IL)-1β
IL-6	interleukin (IL)-6
IL-10	interleukin (IL)-10
NAC	*N*-acetylcysteine
NAD	nicotinamide adenine dinucleotide
NF-κB	nuclear factor kappa B
NQO-1	NAD(P)H and quinone oxidoreductase 1
Nrf2	nuclear factor erythroid 2-related factor 2
ROS	reactive oxygen species
SIRT-1	silent information regulator transcript-1
TNF-α	tumor necrosis factor-α

Conflict of Interest

No potential conflicts of interest were disclosed.

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