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Cytotoxicity Induced by Docetaxel in Human Oral Squamous Cell Carcinoma Cell Lines

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Abstract. *Background: Comparative studies of cytotoxicity of docetaxel against both oral normal and tumor cells are limited. In the present study, the cytotoxicity of docetaxel towards human oral squamous cell carcinoma (OSCC) cell lines (HSC-2, HSC-3, HSC-4) and human oral normal cells (gingival fibroblasts, pulp cells, periodontal ligament fibroblasts) was investigated. Materials and Methods: The cytotoxicity was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide method. Apoptosis induction was monitored by caspase-3/7 activation, DNA fragmentation, and production of annexin-positive cells, and necrosis induction by production of propidium iodide-positive cells. Results: Docetaxel induced various apoptotic markers without loss of the mitochondrial membrane potential and then necrosis markers. When the cells were rinsed with serum-free medium, the cell viability of OSCCs significantly declined. Fetal bovine serum was then fractionated by stepwise ultrafiltration. The growth-promoting activity of fetal bovine serum was heat-stable, and recovered from the fraction >100 kDa. Rinsing with serum-free medium also enhanced the cytotoxicity of docetaxel. Conclusion: The present results suggest that docetaxel may induce apoptosis via a mitochondrial-independent pathway and rinsing of target cells or organs with serum-free medium may reduce the clinical dose of docetaxel and its side effects.*

Docetaxel (Figure 1) is a chemotherapeutic drug classified as a plant alkaloid, a taxane and an antimicrotubule agent (1, 2). Docetaxel has been approved in the treatment of breast cancer, advanced stomach cancer, head and neck cancer and metastatic prostate cancer, and is also being investigated to treat small cell lung, ovarian, bladder, and pancreatic cancer, soft tissue sarcoma and melanoma (3). Docetaxel has been generally used with cisplatin, cisplatin and fluorouracil or carboplatin (4-6) and radiation therapy (7). Pre-operational chemotherapy or radiotherapy combined with docetaxel before extensive surgery for oral squamous cell carcinoma (OSCC) has been used very frequently. However, docetaxel induces side-effects such as neutropenia, neuropathy, severe stomatitis and impairment of liver function (8-10). There are a number of case reports on the clinical usage of docetaxel and research articles on the involved mechanisms in cell death induced by docetaxel in tumor cells except for OSCC cell lines (11, 12). Furthermore, comparative studies of the effects of docetaxel on both oral normal and tumor cells are limited.

In the present study, we investigated the cytotoxicity of docetaxel towards human OSCC cell lines (HSC-2, HSC-3, HSC-4) and human oral normal cells [gingival fibroblast (HGF), pulp cell (HPC), periodontal ligament fibroblast (HPLF)]. In order to reduce the burden on patients during the treatment, we also investigated the minimal treatment time and dose required for induction of cytotoxicity. During the course of experiments, we unexpectedly found that rinsing with serum-free medium enhanced the cytotoxicity of docetaxel. Based on this observation, we partially characterized the responsible serum components.

Materials and Methods

Materials. Docetaxel ($C_{43}H_{53}NO_{14}$, MW 807.93) was purchased from Toronto Research Chemicals, NY, USA and dissolved in dimethyl sulfoxide (DMSO) (Wako Pure Chemical, Osaka, Japan) at 2 mM and stored at –40˚C. RPMI-1640 and Dulbecco's modified Eagle's medium (DMEM) were from Gibco BRL, Grand Island, NY, USA; fetal bovine serum (FBS) from JRH, Bioscience, Lenexa, KS, USA; 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) and phenylmethylsulfonyl fluoride (PMSF) from Sigma Chemical Ind., St. Louis, MO, USA; RNase A, proteinase K, ethidium bromide and agarose S from Nippon Gene Co., Ltd., Toyama, Japan; DNA molecular marker from Bayou Biolabs, Harahan, LA, USA; 6-well, 24-well and 96-microwell plates from Becton Dickinson, Franklin Lakes, NJ, USA; phosphate-buffered

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Key Words: Docetaxel, cytotoxicity, apoptosis, oral squamous cell carcinoma cell lines, serum.

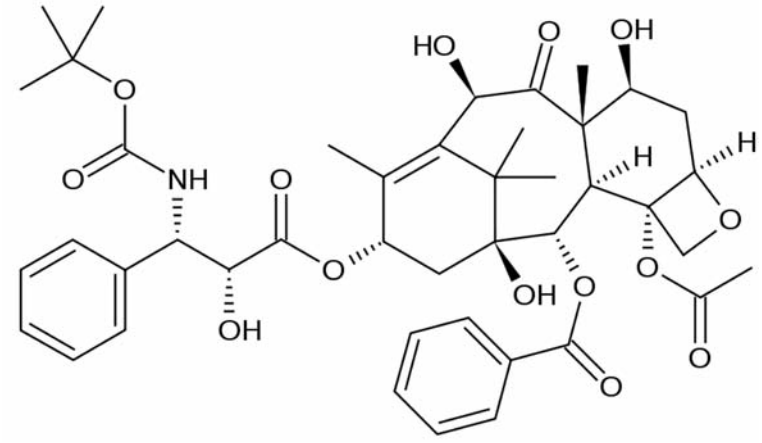

Figure 1. *Structure of docetaxel*.

saline without Mg^{2+} and Ca^{2+} [PBS(−)] from Nissui Pharmaceutical Co., Ltd., Tokyo, Japan; NaI , Hoechst 33258 from Wako Pure Chemical, Caspase Glo 3/7 assay kit from Promega Corporation, Fitchburg, WI, USA; fluorescence photometer from Microlumat LB 96 P, EG&G Berthold, Wildbad, Germany; Ultrafree-MC, NMEL Filter Unit from Millipore Corporation, Bedford, MA, USA, Annexin V-FITC Apoptosis Detection Kit from MBL, Nagoya, Japan; BD Biocoat™ Poly-L-Lysine Cellware 8-well Culture Slide from BD Biosciences, San Jose, CA, USA; CCD camera from Bio Doc-It; UVP, Upland, CA, USA; confocal laser scanning microscope (LMS510) from Carl Zeiss, Gottingen, Germany; plate reader from Multiskan Bichromatic Labsystems, Helsinki, Finland; MitoCapture from Mitochondrial Apoptosis Detection Kit from BioVision, Mountain View, CA, USA.

Cell culture. HL-60 cells (purchased from Riken, Tsukuba, Japan), used as positive control easily committed to apoptosis by UV irradiation, were cultured at 37˚C in RPMI 1640, supplemented with 10% heat-inactivated FBS. Human OSCC cell lines (HSC-2, HSC-3, HSC-2) (purchased from Riken) and normal human oral cells (HGF, HPC, HPLF) [prepared from periodontal tissues, according to the ethic committees of Meikai University School of Dentistry (approved and registered as No. A0808)] were cultured in DMEM supplemented with 10% heat-inactivated FBS. Normal oral cells were used at 8-15 population doubling levels (PDL), since they ceased to proliferate at approximately 20 PDL.

Assay for cytotoxic activity. Viable cell number was determined by the MTT method. Cells were inoculated at 5×10^3 cells/well in 96-microwell plates. After 24 h, the medium was replaced with 0.1 ml of fresh medium containing different concentrations of docetaxel and incubated for 24 or 48 h. The cells were washed once with DMEM supplemented with 10% FBS, then 0.1 ml of medium containing 0.2 mg/ml MTT was added and cells were incubated for 4 h. After removing the medium, 100 μl of DMSO were added to dissolve the formazan dye formed and then the absorbance at 540 nm was measured with a plate reader. The 50% cytotoxic concentration (CC_{50}) was determined from the dose–response curve and the mean value of CC_{50} for each cell type was calculated from 3-6 independent experiments. The tumor-specificity index (TS) was determined by the following equation: $TS=(CC_{50}[HGF]+CC_{50}[HPC]+CC_{50}[HPLF])/(CC_{50}[HSC\text{-}2]+CC_{50}[HSC\text{-}3]+CC_{50}[HSC\text{-}4])$.

Assay for annexin-positive cells. The changes in the localization of membrane phosphatidylserine, an early apoptotic marker, was detected by the Annexin V- fluorescein isothiocyanate (FITC) Apoptosis Detection Kit. Necrosis induction was evaluated by staining with propidium iodide (PI). HSC-2, HSC-3 and HSC-4 cells (15×10^3 cells) were inoculated on Poly-L-Lysine Cellware 8-well Culture Slide and incubated for 24 h. After replenishing with fresh medium, docetaxel (final concentration: 10 nM) was added and cells were incubated for a further 0 (untreated control), 6, 24, 48 or 72 h. The cells were then washed with PBS(−) and then reacted for 15 min at room temperature in the dark with a mixture of a binding buffer (85 μl), Annexin V-FITC (10 μl) and PI (5 μl). After washing once with binding buffer, followed by addition of 200 μl binding buffer, the annexin-positive cells and PI-positive cells were observed at excitation wavelength of 488/543 nm and an emission wavelength of 505-550 nm (green) and >560 nm (red) under a confocal laser scanning microscope. The ratio of annexin V-positive cells to total cells was determined by double staining with Annexin V-FITC and Hoechst 33258. Briefly, cells were reacted for 15 min at room temperature in the dark with a mixture of binding buffer (85 μl), Annexin V-FITC (10 μl) and Hoechst 33258 (50 nM) (4 μl). Cells were washed once with binding buffer, 200 μl binding buffer added, and the annexin-V-positive cells were then observed at an excitation wavelength of 405/488 nm and emission wavelength of 505-550 nm (green) and 420-480 nm (blue), under a confocal laser scanning microscope. The ratio of Hoechst 33258-positve cells (stained blue) to annexin V-positive cells (stained green) was determined in 20 different fields per sample.

Assay for caspase activity. HSC-2, HSC-3 and HSC-4 cells were inoculated at 5×10^3 cells/well in a 96-microwell plate. After 24 h, the medium was replaced with 0.1 ml of fresh medium containing different concentrations of docetaxel and incubated for 24 or 48 h. After removing the medium, cells were reacted for 30 min at room temperature with 100 μl of buffer containing substrate having

DEVD sequence (Caspase Glo 3/7 assay kit) and the fluorescence intensity of the cleaved product was measured by a fluorescence photometer.

Assay for DNA fragmentation. HSC-2, HSC-3 and HSC-4 cells were inoculated at 5×10^4 cells/well in 24-well plates. After 24 h, the medium was replaced with 1 ml of fresh medium containing different concentrations of docetaxel and incubated for 72 h. Cells were scraped with a rubber policeman and collected by centrifugation at 4˚C for 20 min at 1,800 ×*g*, and washed once with PBS(–). The pelleted cells were lysed by lysate buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroylsarcosinate]. The lysate was incubated with 0.4 mg/ml RNase A and 0.8 mg/ml proteinase K for 2 h at 50˚C, and then mixed with 50 μl NaI solution [7.6 M NaI, 20 mM EDTA-2Na, 40 mM Tris-HCl, pH 8.0] and 100 μl of ethanol. After centrifugation for 20 min at 20,000 ×*g*, the precipitate was washed with 1 ml of 70% ethanol, dissolved in 12 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 3-5), and then applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0). The DNA molecular marker (Takara, Shiga, Japan) and the DNA from apoptotic HL-60 cells induced by ultraviolet (UV) irradiation (6 J/m^2/min, 1 min) were used for calibration. DNA was stained with ethidium bromide (0.5 μg/ml) and the DNA fragmentation pattern was examined in photographs taken under UV illumination.

Assay for membrane potential of mitochondria. The Mitocapture Mitochondrial Apoptosis Detection Kit was used to detect the membrane potential of mitochondria. HSC-2, HSC-3 and HSC-4 cells were inoculated at 15×10^3 cells onto Poly-L-Lysine Cellware 8-well Culture Slide and incubated for 24 h. After replenishing with fresh medium, docetaxel (final concentration: 10 nM) was added and cells were incubated for a further 0 (untreated control), 6, 24, 48 or 72 h. After washing with PBS(–), the cells were reacted for 15 min at 37˚C with Mitocapture (diluted 1:1000 with incubation buffer) (Mitocapture Mitochondrial Apoptosis Detection Kit) in 5% CO_2 incubator. Cells were washed with incubation buffer, 200 μl incubation buffer added and the mitochondrial membrane potential was then measured at excitation wavelength of 488/543 nm and emission wavelength of 505-530 nm (green) and >560 nm (red) under a confocal laser scanning microscope. As positive control, apoptotic HSC-3 cells induced by treatment for 3 h with actinomycin-D (1 μM) were used.

Fractionation of FBS by ultrafiltration. Three filters [Ultrafree-MC (10,000, 30,000, 100,000)] were used to fractionate the FBS components. FBS (200 μl) was centrifuged for 10 min at 4˚C, 4500 ×*g* through MC100,000 to retain the substances larger than 100 kDa. The filtrate was applied with centrifugation to MC 30,000 to retain substances between 30-100 kDa. The filtrate was then applied with centrifugation to MC 10,000 to retain substances between 10-30 kDa, and recover those smaller than 10 kDa. All of these separated fractions were mounted to 200 μl in volume and added to DMEM at a final concentration of 10%.

Effect of rinsing with serum-free medium on the recovery of viable cells. HSC-2, HSC-3 and HSC-4 cells were inoculated at 5×10^3 cells/well in 96-microwell plate. The cells during the culture were rinsed with (i) serum-free PBS (-) or DMEM, (ii) PBS (-) or DMEM containing 10% FBS, (iii) DMEM containing FBS fractions prepared by ultrafiltration, or heat-denatured (100˚C, 5 min) FBS, and the viability of the cells was then determined by MTT method.

Table I. *Cytotoxic activity of docetaxel towards human oral cells and human oral squamous cell carcinoma cell lines. Data are means±S.D. from three independent experiments performed in triplicate.*

Cells	CC_{50} (nM) of docetaxel
Human normal oral cells	
Gingival fibroblasts	>100
Pulp cells	>100
Periodontal ligament fibroblasts	>100
Human oral squamous cell carcinoma cell line	
HSC-2	10.7±2.3
HSC-3	5.7±1.0
HSC-4	6.3±3.1

CC_{50}: 50% cytotoxic concentration.

Statistical treatment. The difference between groups was evaluated by Student's *t*-test. The value of statistical significance was set at the 0.05 level.

Results

Cytotoxicity of docetaxel towards normal and tumor cells. Docetaxel showed a dose-dependent cytotoxicity towards human OSCC cell lines (HSC-2, HSC-3, HSC-4). These cells showed comparable sensitivity at 24 h after treatment (Figure 2A). Upon treatment for 48 h with 10-100 nM docetaxel, the viable cell number declined to 40-50% that of the control (Figure 2B). Cytotoxicity of docetaxel was not significantly changed even when the concentration was increased to 10 μM (Figure 2C). HSC-3 cells exhibited the highest sensitivity to docetaxel (CC_{50}=5.7 nM), followed by HSC-4 (CC_{50}=6.3 nM) and then HSC-2 (CC_{50}=10.7 nM). The mean value of CC_{50} for the OSCC cell lines was calculated as 7.6 nM. On the other hand, normal human oral cells (HGF, HPC, HPLF) exhibited much lower sensitivity towards docetaxel (CC_{50} >100 nM) (Figure 2D). From these CC_{50} values, the TS was calculated to be >13.2 (Table I).

Apoptosis induction by docetaxel. We investigated whether docetaxel induces apoptosis. The production of annexin-positive cells (visualized by green color) was increased in HSC-2 cells 6 h after treatment with docetaxel, and 24 h after treatment in HSC-3 and HSC-4 cells, whereas the production of PI-positive necrotic cells (visualized by red color) was increased at 72 h after treatment with docetaxel (Figure 3A). Next, the ratio of annexin-positive cells to the total cells was calculated by double-staining with Hoechst 33258 (which stains nuclei) and annexin V-FITC (Figure 3B) and quantified by image analysis (Figure 3C). For HSC-2

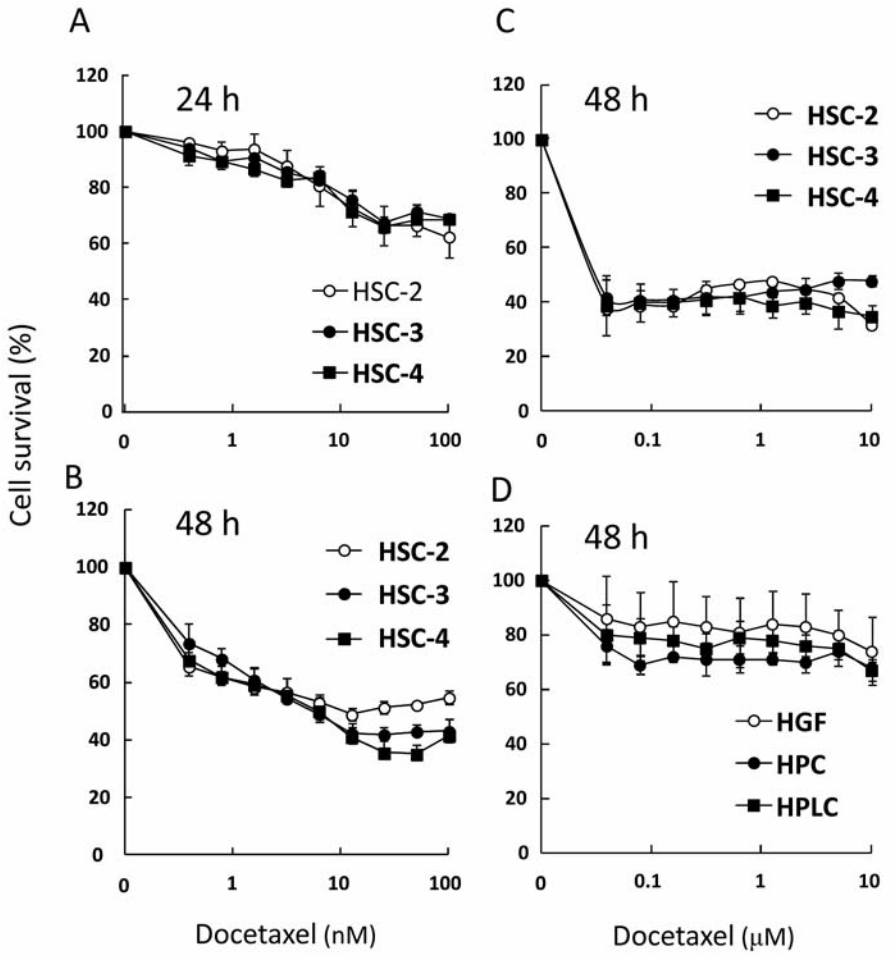

Figure 2. *Cytotoxic activity of docetaxel towards human oral squamous cell carcinoma (OSCC) cell lines (A, B, C) and human oral normal cells (D) at nanomolar (A, B) and micromolar (C, D) concentrations. The cells were incubated for 24 (A) or 48 (B, C, D) h with the indicated concentrations of docetaxel. The viable cell number was then determined by the 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) assay, and expressed as a percentage to that of the control. Each point represents the mean±S.D. for three independent experiments.*

cells, annexin-positive cells appeared 6 h after docetaxel treatment, and increased with an incubation time up to 48 h. For HSC-3 cells, a similar time-dependent increase in annexin-positive cells was observed, but to a lesser extent. For HSC-4 cells, the marked appearance of annexin-positive cells was detected at 24-48 h (Figure 3C). Under phase-contrast microscopy, docetaxel treatment for 48 h was found to produce multi-nucleated HSC-3 cells (Figure 3D).

When HSC-2, HSC-3 and HSC-4 cells were incubated for 24 h with 10 nM docetacel, caspase-3/7 activity was increased by 3.2-, 3.5- and 3.1-fold, respectively, compared to the untreated control (Figure 4A). Similar magnitude of caspase activation was observed 48 h after treatment with 1 nM docetaxel (Figure 4B).

Treatment with docetaxel (1, 10 nM) for 72 h induced inter-nucleosomal DNA fragmentation in HSC-4 cells, but not in HSC-2 and HSC-3 cells (Figure 5).

The possible involvement of the mitochondrial apoptotic pathway was investigated using a Mitocapture Mitochondrial Apoptosis Detection Kit (Figure 6). This probe emits red fluorescence in the mitochondria when the mitochondrial membrane is intact, but emits green fluorescence in the cytosol when the mitochondrial membrane is injured (13). When HSC-2, HSC-3, HSC-4 cells were treated for 6-48 h with docetaxel, mitochondria emitted a red color, but not a green color. On the other hand, actinomycin-D (1 μM, 3 h)-treated cells, used as positive control, exhibited a green color (Figure 6). These data suggest that docetaxel does not reduce the mitochondrial membrane potential.

Minimum treatment time required for cytotoxicity induction. The intensity of cytotoxicity induced by docetaxel was compared between the following two treatment conditions: (i) Cells were continuously treated for 48 h with docetaxel;

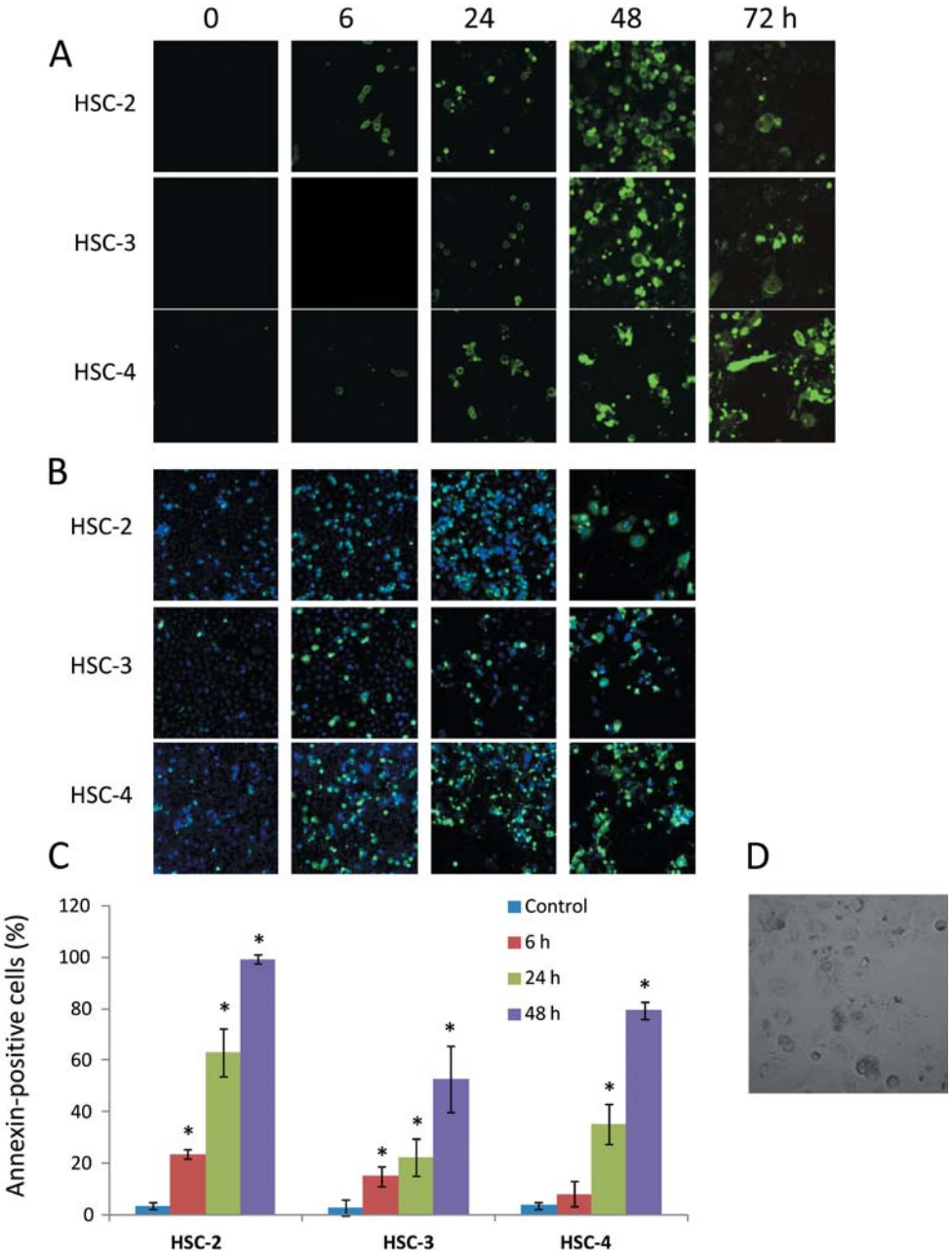

Figure 3. *Increase in annexin V-positive cells by treatment with docetaxel. HSC-2, HSC-3 and HSC-4 cells were either left untreated (0 h) or were treated with docetaxel (10 nM) for the indicated periods (6-72 h). After staining with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) (A) or annexin V-FITC and Hoechest 33258 (B), cells were observed under confocal laser scanning microscopy. Original magnification ×20. The percentage of annexin V-positive HSC-2, HSC-3 and HSC-4 cells was determined (C). Phase-contrast microscopy image of HSC-3 cells (D). *Significant difference ($p<0.05$) from untreated control (0 h).*

and (ii) cells were first treated for 1, 2 or 3 h with docetaxel, washed with FBS-containing medium and then re-cultured for 47, 46, or 45 h in docetaxel-free medium (Figure 7A). When the concentration of docetaxel was above the CC_{50} value (10.7 nM), the cytotoxicity in HSC-2 cells was comparable between 1 h of treatment and 48 h of treatment (Figure 7B). On the other hand, cytotoxicity of docetaxel significantly declined with diminishing treatment time (3 h < 2 h < 1 h) in HSC-3 and HSC-4 cells (Figure 7C and D). When the treatment time with docetaxel was extended to 6 or 12 h, the intensity of cytotoxicity reached a level comparable to that attained by 48 h of treatment (Figure 7E-

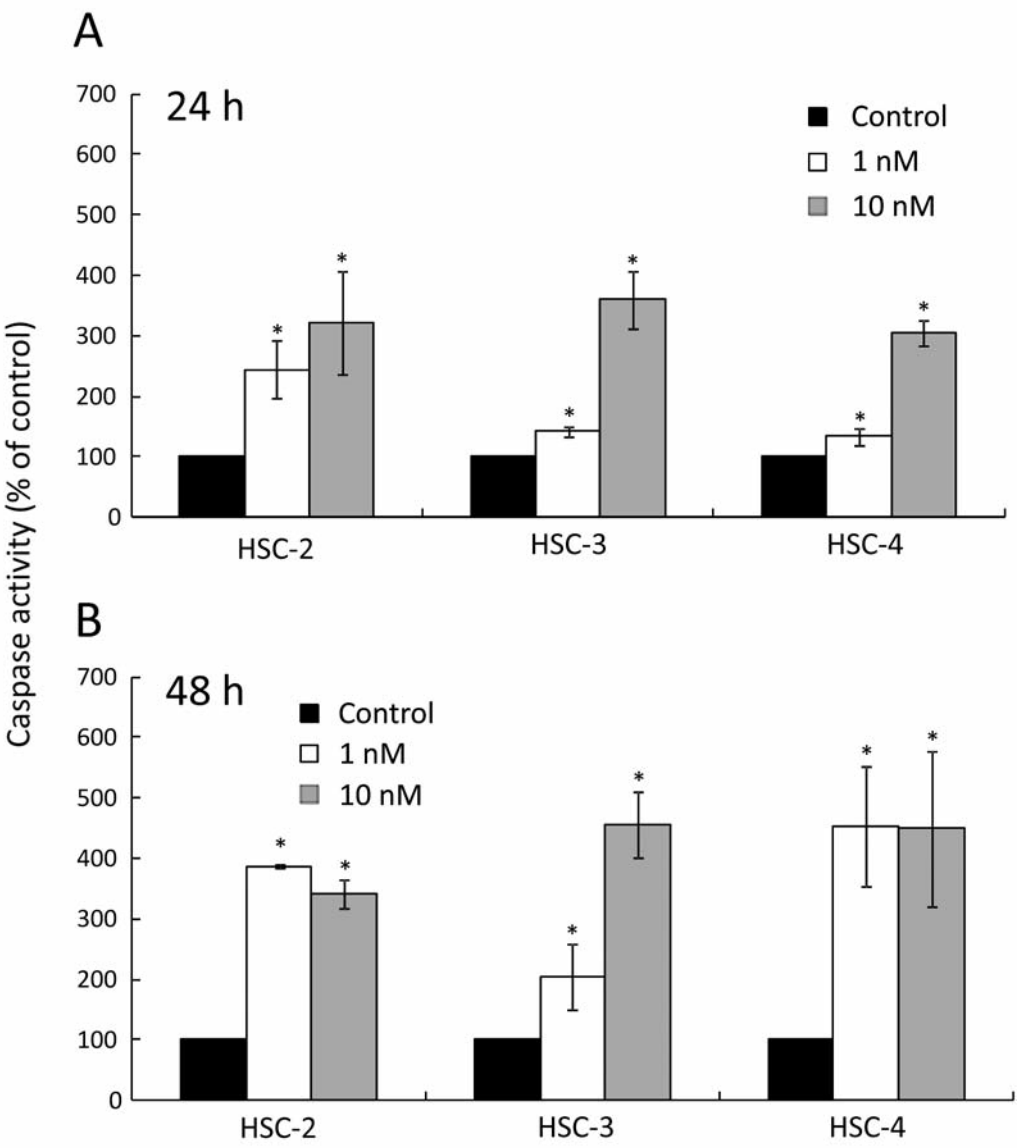

Figure 4. *Activation of caspase-3 by docetaxel in human oral squamous cell carcinoma (OSCC) cell lines. HSC-2, HSC-3 and HSC-4 cells were either left untreated (control) or treated with docetaxel (1 or 10 nM) for 24 (A) or 48 h (B), and caspase-3 activity was then determined by substrate cleavage assay. Each symbol and bar represents the mean±S.D. from three independent experiments performed in triplicate. *Significant difference (p<0.05) from control.*

G). These data demonstrate that the minimum treatment time required for cytotoxicity induction by docetaxel is 1 h for HSC-2 cells and 6 h for HSC-3 and HSC-4 cells.

Effect of rinsing with serum-free medium on cytotoxicity assessment. HSC-2, HSC-3, HSC-4 cells were cultured for 24 h. The cells were then rinsed once with DMEM with or without FBS, and then incubated for 48 h in DMEM supplemented with FBS to determine the viable cell number by the MTT method (Figure 8A). Rinsing with FBS-free DMEM resulted in a 20-30% decrease in the viable cell number as compared with that with FBS-containing DMEM (Figure 8B). Rinsing with FBS-free PBS(–) similarly reduced the viability as compared with that with FBS-containing PBS(–) (Figure 8B).

On the other hand, rinsing with FBS-free PBS(–) did not reduce the number of viable cell number of normal cells (HGF, HPC, HPLF), in contrast to HSC-2 cells (Figure 8C). These results show that some components of FBS appear to be necessary to prevent the loss of viability of OSCC cell lines during the rinsing step, but not of normal oral cells. The protective effect of FBS was not lost by heat treatment

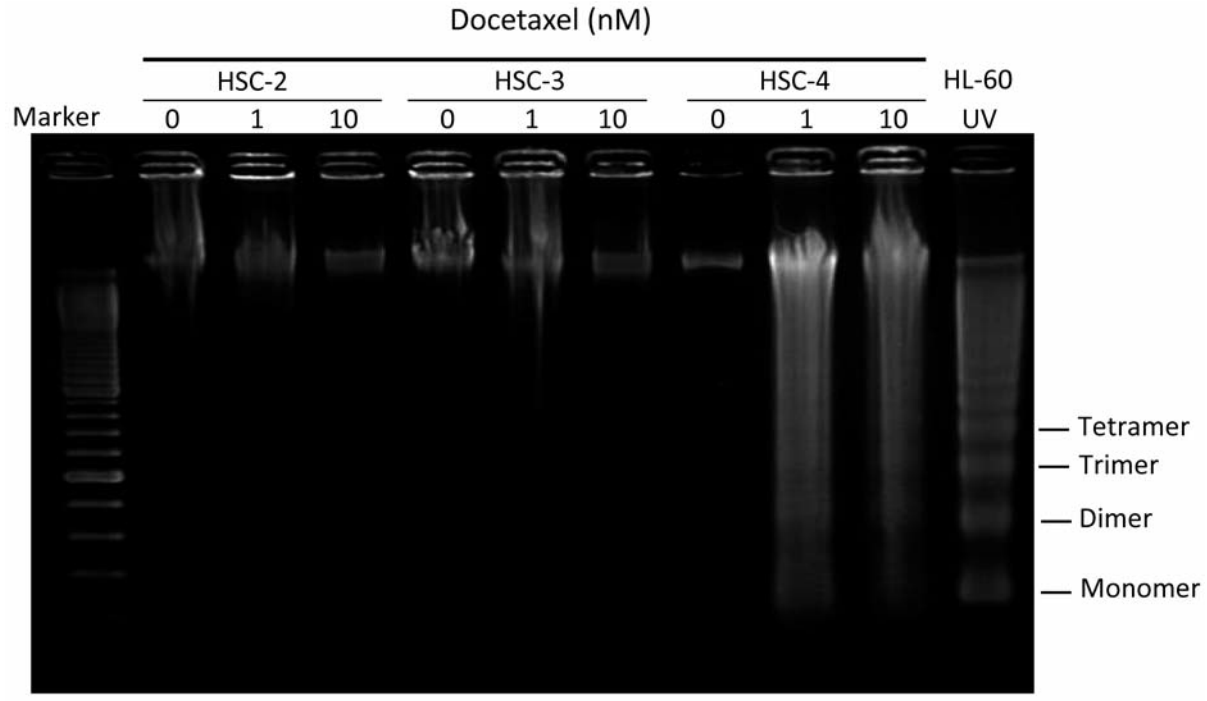

Figure 5. *Induction of DNA fragmentation by docetaxel in human oral squamous cell carcinoma (OSCC) cell lines. HSC-2, HSC-3 and HSC-4 cells were either left untreated (0 nM) or treated with docetaxel (1 or 10 nM) for 72 h. DNA was then extracted and subjected to agarose gel electrophoresis. M, DNA marker; UV, DNA from apoptotic HL-60 cells induced by UV-irradiation.*

(100˚C, 5 min) (Figure 8D). Rinsing with DMEM containing the highest molecular weight FBS fraction (>100 kDa) led to a comparable magnitude of protection with that when washed by FBS-containing DMEM, whereas rinsing with DMEM containing lower molecular weight FBS fractions (<10 kDa, 10-30 kDa, 30-100 kDa) resulted in 30-40% decrease in viable cell numbers (Figure 8E).

Effect of washing with FBS-free medium on the cytotoxic action of docetaxel. HSC-2, HSC-3 and HSC-4 cells were cultured for 24 h. Cells were then incubated for 6 h with docetaxel (0, 1, 10 nM), rinsed with either FBS-free DMEM or FBS-containing DMEM, and cultured for 42 h in fresh FBS-containing DMEM to determine the viable cell number by the MTT method (Figure 9A). Rinsing with FBS-free DMEM significantly ($p<0.05$) augmented the cytotoxicity of docetaxel (1 nM), as compared with rinsing with FBS-containing DMEM for all the cell lines (Figure 9B). This augmenting effect of rinsing with FBS-free DMEM was comparable with that observed when a 10-fold higher concentration (10 nM) of docetaxel was used. Since these phenomena were observed for HSC-2, HSC-3 and HSC-4 cells, rinsing with serum-free medium reduced the required administration dose of docetaxel to achieve cytotoxicity to one tenth.

Discussion

The present study demonstrated that docetaxel exhibited higher cytotoxicity towards human OSCC cell lines as compared with normal oral cells. Considering that OSCC cell lines have shorter doubling time (approximately 24 h), as compared with normal oral cells (approximately 36 h), the cytotoxic action of docetaxel may depend on the cell-cycle progression. HSC-2 and HSC-3 cells have mutated *p53* tumor-suppressor gene (14-18). Overexpression of p53R2 (p53-dependent DNA repair enzyme) resulted in the reduction of sensitivity towards anticancer drugs in HSC-4 cells (19). We found that HSC-2, HSC-3 and HSC-4 cells exhibited comparable sensitivity to docetaxel, and therefore that a p53-independent mechanism may be involved in the cytotoxicity induction by docetaxel. This is supported by a previous finding that apoptosis induction by docetaxel was p53-independent (20).

The present study demonstrated that docetaxel first induced the production of apoptosis marker such as the production of annexin-positive cells and caspase activation (6~24 h after treatment), and then necrosis markers such as production of PI-positive cells (72 h after treatment). Docetaxel may induce apoptosis *via* a mitochondria-independent pathway, considering that a loss of mitochondrial membrane potential

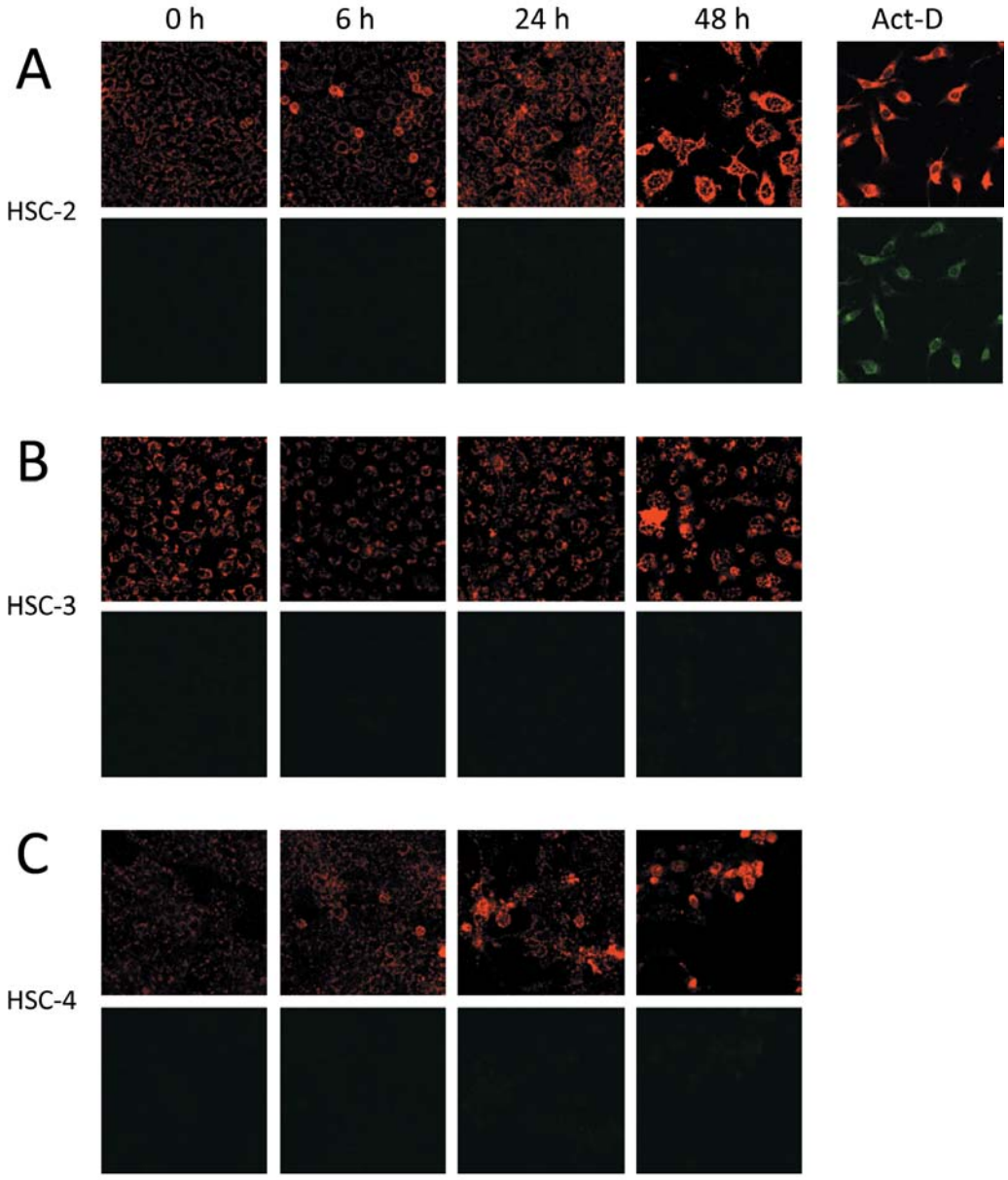

Figure 6. *Effect of docetaxel on the mitochondria membrane potential. HSC-2 (A), HSC-3 (B) and HSC-4 (C) cells were either left untreated (0 h) or treated with docetaxel (10 nM) for the indicated periods (6-48 h). HSC-2 cells treated for 3 h with actinomycin-D (ActD; 1 μM) were used as a positive control. After staining with Mitocapture, cells were observed under confocal laser scanning microscopy. Original magnification, ×20.*

was not observed. This result is inconsistent with previous finding that docetaxel induced mitochondrial-mediated apoptosis in HSC-3 cells (21). These contradictory results may be due to the different experimental conditions: the previous study used scraped cells, which may lead to the damage of cells (21), while the present study used intact attached cells.

In order to reduce the toxicity of anticancer drugs to normal cells, a minimum treatment time of docetaxel should be determined. The present study demonstrated that the treatment time of docetaxel can be reduced to 1 h for HSC-2 cells, and to 6 h, without reducing the intensity of cytotoxicity. Similar time-course experiments with animals are required.

As a by-product of the present study, we found that rinsing with FBS-free medium significantly and reproducibly reduced the viability of OSCC cells, but not that of normal oral cells. This phenomenon was not simply due to cell detachment during the rinsing procedure, since cell counting

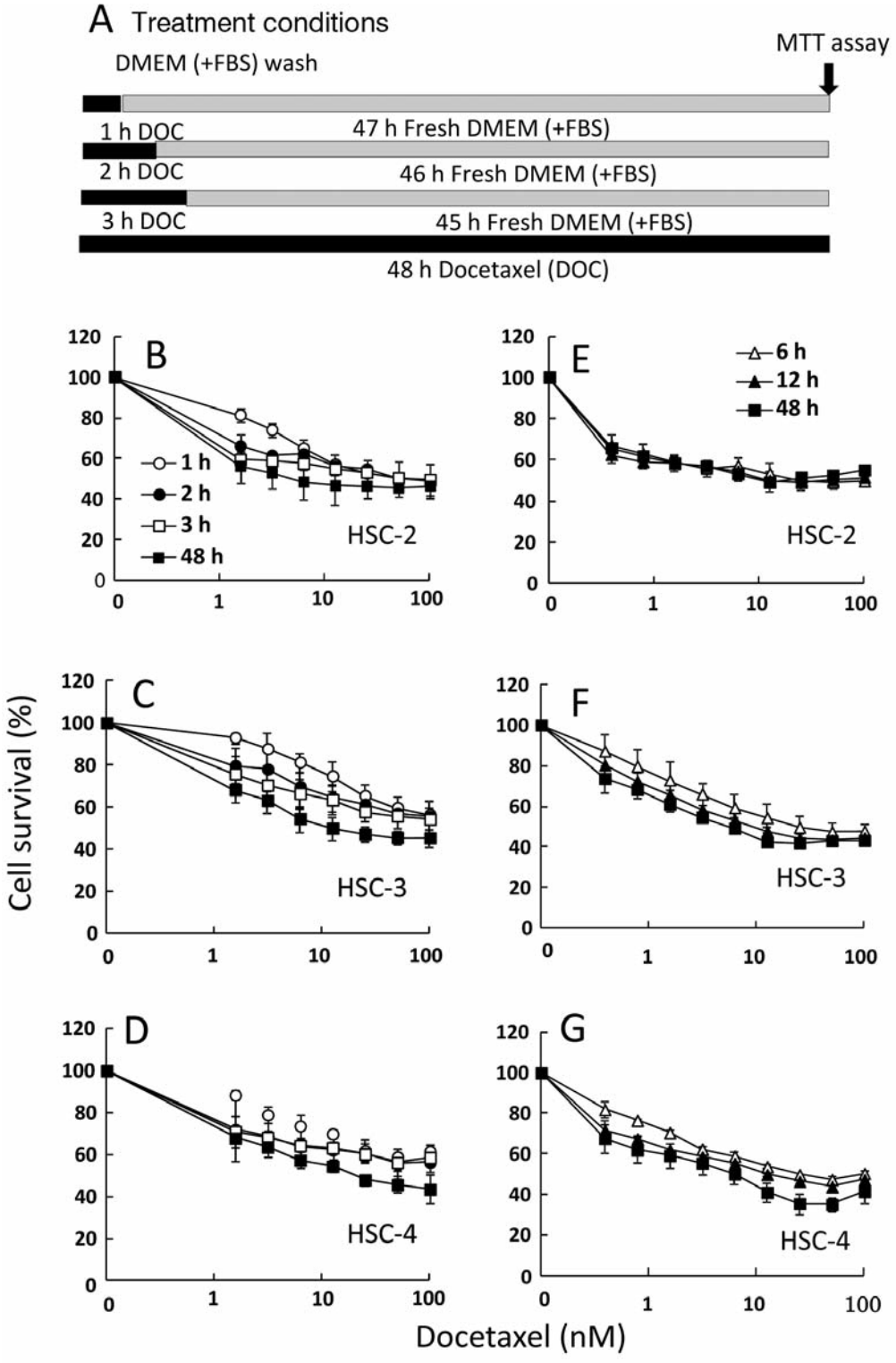

Figure 7. *Determination of optimal treatment time with docetaxel. Experimental procedure (A). HSC-2 (B, E), HSC-3 (C, F) and HSC-4 (D, G) cells were treated with different concentrations of docetaxel for 1-3 h (B, C, D), 6-12 h (E, F, G), or continuously treated for 48 h (B-G). The cells treated for 1-12 h with docetaxel were rinsed with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), and then incubated for 47-36 h without docetaxel, maintaining a total incubation time of 48 h. The relative cell viability was then determined by the MTT assay. Each symbol represents the mean±SD of three independent experiments performed in triplicate.*

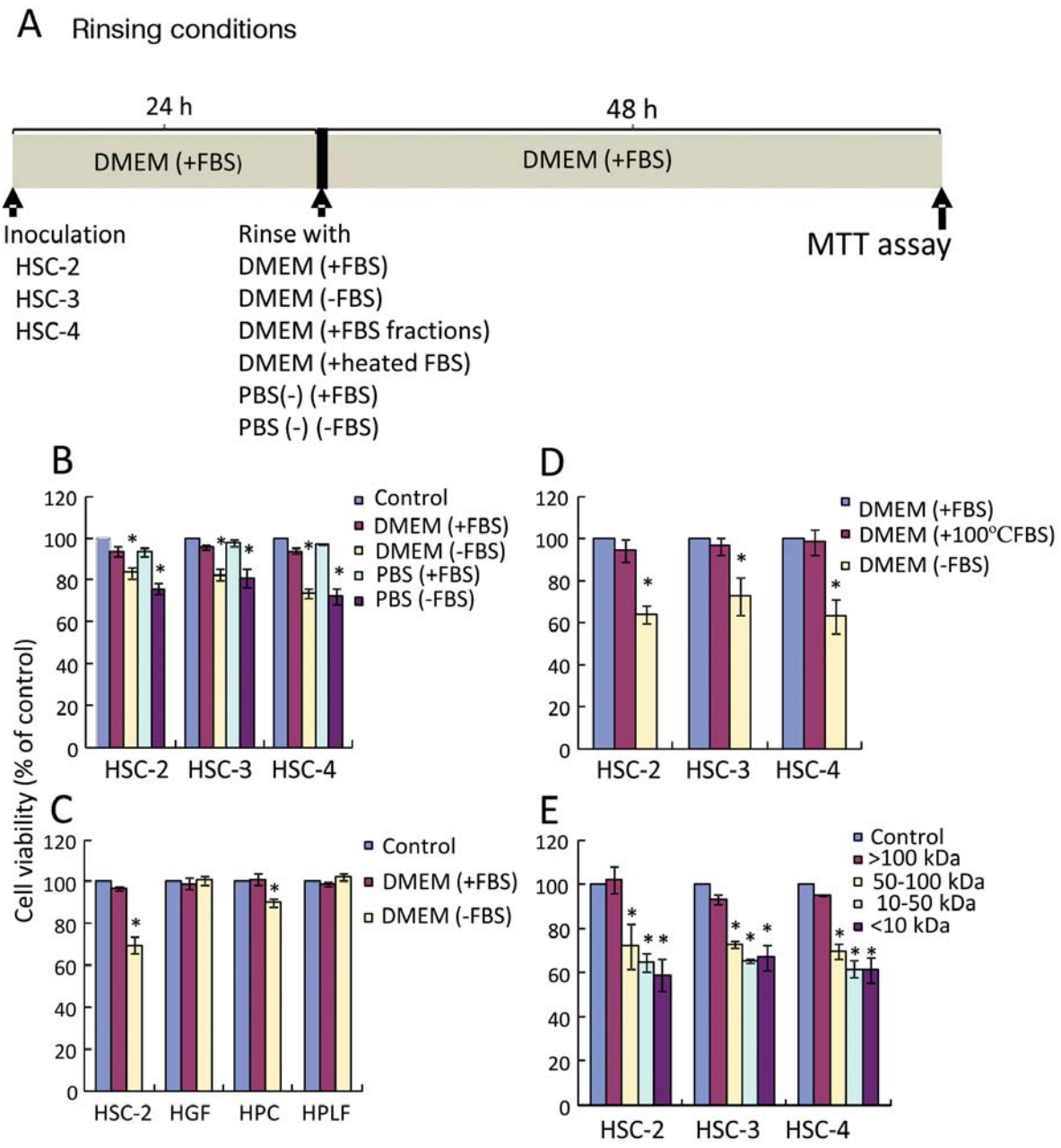

Figure 8. *Rinsing without serum reduced the viability of human oral squamous cell carcinoma (OSCC) cell lines. Experimental procedure (A). Human OSCC cell lines (B, D, E) and human normal oral cells (C) were cultured in regular culture medium for 24 h. Cells were not rinsed (control) or rinsed with DMEM or phosphate-buffered saline without Mg^{2+} and Ca^{2+} [PBS(−)] in the presence or absence of fetal bovine serum (FBS) (B, C), heated FBS (D) or FBS fractions separated by successive ultrafiltrations (E), and then incubated in regular culture medium for 48 h. Cell viability was determined by MTT assay. Each symbol represents the mean±SD of three independent experiments performed in triplicate. *Significant difference ($p<0.05$) from control (without rinsing).*

immediately after rinsing showed no difference in the residual cell numbers (data not shown). The present study demonstrated that the serum component(s) that prevented the loss of viable cells during rinsing were easily washable, heat-stable and had a high molecular weight. These unique properties suggest that these component(s) may be water-soluble, and might not be enzymes, most of which are heat-sensitive.

As far as we know, there is no similar report regarding the cytoprotective effect of FBS. This unexpected finding may

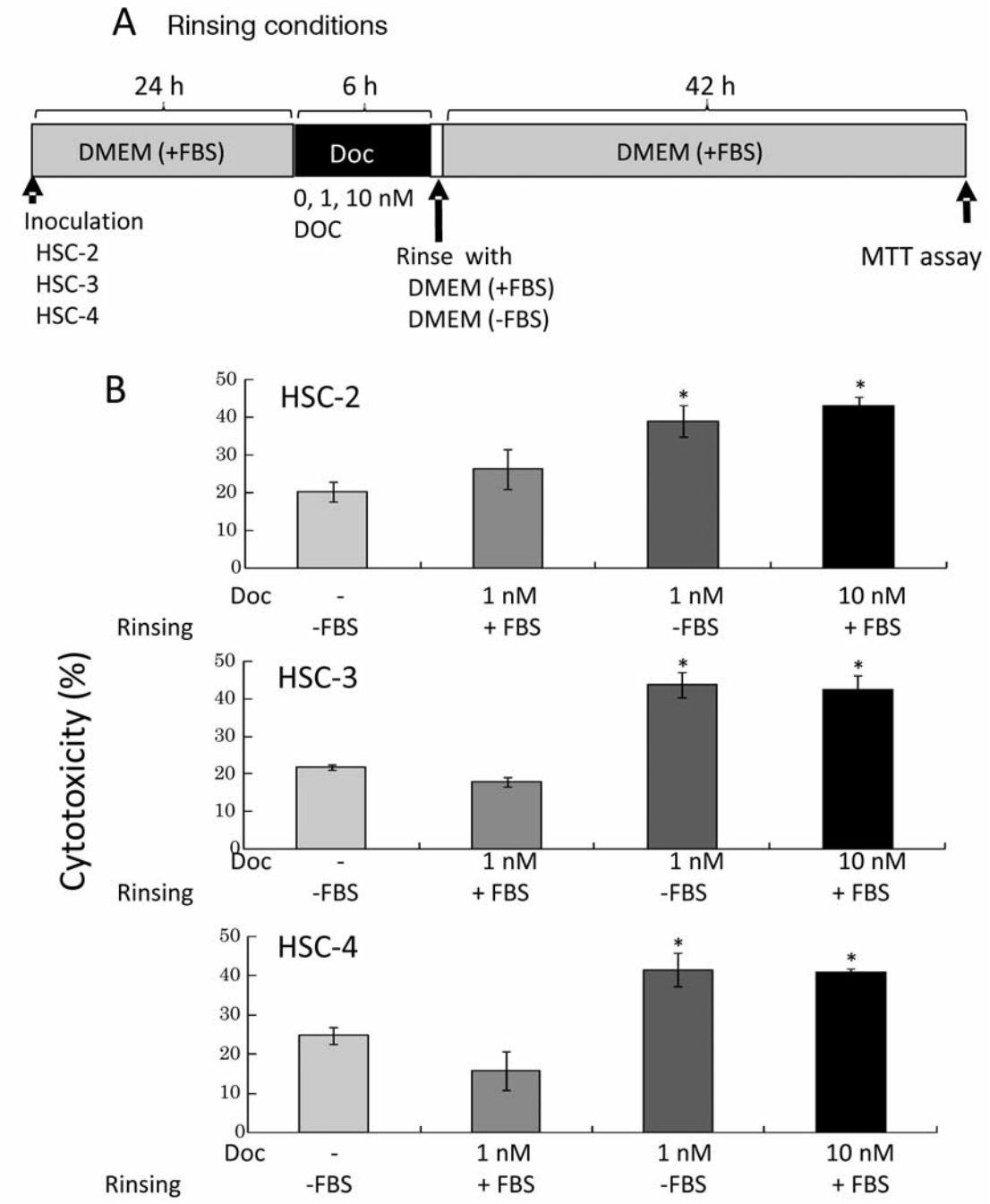

Figure 9. *Augmentation of docetaxel cytotoxicity by subsequent rinsing without fetal bovine serum (FBS). Experimental procedure (A). HSC-2, HSC-3 and HSC-4 cells were either left untreated or treated with docetaxel (Doc) (1 or 10 nM) for 6 h before rinsing with Dulbecco's modified Eagle's medium (DMEM) that contained 0 or 10% FBS. Cells were then incubated in regular culture medium containing 10% FBS for 42 h (B). Relative cell viability was determined by MTT assay. Each symbol represents the mean±S.D. of three independent experiments performed in triplicate. *Significant difference ($p<0.05$) from washing of 1 nM docetaxel-treated cells with FBS-free DMEM.*

have biological implications in cancer cell-based research in general. The MTT assay is frequently used by many cancer researchers without paying attention to the effect of FBS. It is strongly recommendable that FBS should be included in washing buffer for the MTT assay. Without FBS, the viable cell number would be underestimated.

In conclusion, the present results suggest that docetaxel may induce apoptosis *via* a mitochondrial-independent

pathway and rinsing of target cells or organs with serum-free medium may reduce the clinical dose of docetaxel and its side-effects.

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References

1 Lavelle F, Bissery MC, Combeau C, Riou JF, Vrignaud P and Andre S: Preclinical evaluation of docetaxel. Semin Oncol *22*: 3-16, 1995.
2 Kaplow R: Innovations in antineoplastic therapy. Review. Nurs Clin North Am *40*: 77-94, 2005.
3 Eisenhauer EA and Vermorken JB: The taxoids: Comparative clinical pharmacology and therapeutic potential. Drugs *55*: 5-30, 1998.
4 Forastiere AA, Metch B, Schuller DE, Ensley JF, Hutchins LF, Triozzi P, Kish JA, McClure S, VonFeldt E, Williamson SK and VonHoff DD: Randomized comparison of cisplatin plus fluorouracil and carboplatin plus fluorouracil *versus* methotrexate in advanced squamous-cell carcinoma of the head and neck: a Southwest Oncology Group study. J Clin Oncol *10*: 1245-1251, 1992.
5 De Andres L, Brunet J, Lopez-Pousa A, Burgues J, Vega M, Tabernero JM, Mesia R and Lopez JJ: Randomized trial of neoadjuvant cisplatin and fluorouracil *versus* carboplatin and fluorouracil in patients with stage IV-M0 head and neck cancer. J Clin Oncol *13*: 1493-1500, 1995.
6 Schrijvers D, Van Herpen C, Kerger J, Joosens E, Van Laer C, Awada A, Van den Weyngaert D, Nguyen H, Le Bouder C, Castelijns JA, Kaandera J, De Mulder P and Vermorken JB: Docetaxel, cisplatin and 5- fluorouracil in patients with locally advanced unresectable head and neck cancer: A phase I-II feasibility study. Ann Oncol *15*: 638-645, 2004.
7 Herskovic A, Martz K, Sarraf M, Leichman L, Brindle J, Vaitkevicius V, Cooper J, Byhardt R, Davis L and Emami B: Combined chemotherapy and radiotherapy compared with radiotherapy alone in patients with cancer of the esophagus. N Engl J Med *326*: 1593-1598, 1992.
8 Yared JA and Tkaczuk: Update of taxane development: New analogues and new formulations. Drug Des Devel Ther *6*: 371-384, 2012.
9 Raisch DW, Campbell W, Garg V, Qureshi ZP, Bookstaver PB, Norris and Bennett CL: Description of anaphylactic reactions to paclitaxel and docetaxel reported to the FDA, with a focus on the role of premedication. Expert Opin Drug Saf *10*: 521-528, 2011.
10 Muro K, Hamaguchi A and Ohtsu N: A phase II study of single-agent docetaxel in patients with metastatic esophageal cancer. Annal Oncol *15*: 955-959, 2004.
11 Katsumata N, Noda K, Nozawa S, Kitagawa R, Nishimura R, Yamaguchi S, Aoki D, Susumu N, Kuramoto H, Jobo T, Ueki K, Ueki M, Kohno I, Fujiwara K, Sohda Y and Eguchi F : Phase II trial of docetaxel in advanced or metastatic endometrial cancer: A Japanese Cooperative Study. Br J Cancer *93*: 999-1004, 2005.
12 Braakhuis BJ, Hill BT, Dietel M, Kelland LR, Aapro MS, Zoli W and Lelieveld P: *In vitro* antiproliferative activity of docetaxel (Taxotere), paclitaxel (Taxol) and cisplatin against human tumour and normal bone marrow cells. Anticancer Res *14*: 205-208, 1994.
13 Belkhiri A, Zaika A, Pidkovka N, Knuutila S, Moskaluk C and El-Rifai W: DARPP-32: A novel antiapoptotic gene in upper gastrointestinal carcinomas. Cancer Res *65*: 6583-6592, 2005.
14 Tachibana M, Shinagawa Y, Kamamata H, Omotehara F, Horiuchi H, Ohkura Y, Kubota K, Imai Y, Fujibayashi T and Fujimori T: RT-PCR amplification of RNA extracted from formalin-fixed, paraffin-embedded oral cancer sections. Anticancer Res *23*: 2891-2896, 2003.
15 Kamiya Y and Ohshima T: The Individual cell properties of oral cell carcinoma and p53 tumor suppressor gene mutation. Oral Sci Int *2*: 104-117, 2005.
16 Kaneda Y, Shimamoto H, Matsumura K, Arvind R, Zhang S, Sakai E, Omura K and Tsuchida N: Role of caspase 8 as a determinant in chemosensitivity of p53-mutated head and neck squamous cell carcinoma cell lines. J Med Dent Sci *53*: 57-66, 2006.
17 Sakai E and Tsuchida N: Most human squamous cell carcinomas in the oral cavity contain mutated *p53* tumor-suppressor genes. Oncogene *7*: 927-933, 1992.
18 Kashiwazaki Y: Detectability and diagnostic criteria of *p53* gene mutations in human oral squamous cell carcinoma using yeast functional assay. Hokkaido Igaku Zasshi *72*: 211-224, 1997 (in Japanese).
19 Yanamoto S, Iwamoto T, Kawasaki G, Yoshitomi I, Baba N and Mizuno A: Silencing of the *p53R2* gene by RNA interference inhibits growth and enhances 5-fluorouracil sensitivity of oral cancer cells. Cancer Lett *223*: 67-76, 2005.
20 Schimming R, Manson KA, Hunter N, Weil M, Kishi K and Milas L: Lack of correlation between mitotic arrest or apoptosis and antitumor effect of docetaxel. Cancer Chemother Pharmacol *43*: 165-172, 1999.
21 Taniguchi T, Takahashi M, Shinohara F, Sato T, Echigo S and Rikiishi H: Involvement of NF-κB and mitochondrial pathways in docetaxel-induced apoptosis of human oral squamous cell carcinoma. Int J Mol Med *15*: 667-673, 2005.

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