Effects of Reactive Oxygen Species on Cell Proliferation and Death in HeLa Cells and Its MDR1-Overexpressing Derivative Cell Line

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In this paper, the effects of H2O2, a typical reactive oxygen species (ROS), on cell proliferation or death were examined using the human cervical carcinoma cell line HeLa and its MDR1-overexpressing subline, Hvr100-6, which was established by stepwise exposure to vinblastine. It was confirmed that the growth of HeLa cells was enhanced by H2O2 at relatively low concentrations in a concentration-dependent manner, and the growth enhancement was suppressed by antioxidants. Doxorubicin and daunorubicin also enhanced the growth of HeLa cells at concentrations lower than IC50 values, and the antioxidants suppressed this effect, being consistent with the fact that both anticancer drugs generate ROS. The growth enhancement by H2O2 or doxorubicin and daunorubicin was not observed in Hvr100-6 cells. In addition, it was suggested that antioxidants had no effect on MDR1 mRNA expression in HeLa and Hvr100-6 cells, and thereby hardly reverse multidrug resistance in tumor cells.

Key words MDR1; P-glycoprotein; antioxidant; reactive oxygen species (ROS); HeLa cell

Reactive oxygen species (ROS) have been recognized to pose a constant threat to cells as they can severely damage DNA, protein and lipids, despite that they are produced endogenously or derived from external sources. However, recent investigations on the oxidative stress response mechanisms have suggested that oxidative stress can result in a wide spectrum of responses at the cellular level from proliferation to growth arrest and death.2,3) Recently, it has been demonstrated that ROS production is one common signaling event in the course of signal transduction initiated by cytokines or growth factors,4) and that doxorubicin and daunorubicin generate ROS.5,6) These results suggest that both anticancer drugs enhance tumor growth under certain conditions, although there is no such clinical evidence.

Much experimental evidence indicates that both the tumor suppressor p53 and the multidrug resistant transporter MDR1 play important roles in chemoresistance.7,8) MDR1 is a glycosylated membrane protein of 170 kDa, belonging to the ATP-binding cassette superfamily, and acts as an energy-dependent efflux pump that exports substrates out of cells.9–11) Numerous clinical studies have concluded that MDR1 overexpression was associated with a failure of chemotherapy; however, there is no information on the relationship between the effects of oxidative stress on cell proliferation or death and MDR1 expression levels.

In this paper, the effects of ROS, H2O2 or catalase inhibitor (3-amino-1,2,4-triazole) on cell proliferation or death were examined using the human cervical carcinoma cell line HeLa and its MDR1-overexpressing subline, Hvr100-6, which was established by stepwise exposure to vinblastine.12) The effects of doxorubicin and daunorubicin were also evaluated.

MATERIALS AND METHODS

Chemicals L(+)-Ascorbic acid and hydroquinone were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Baicalein, genistein, quercetin and 3-amino-1,2,4-triazole were obtained from Sigma-Aldrich Chemical (St. Louis, MO, U.S.A.). Vinblastine sulfate, paclitaxel, doxorubicin hydrochloride, daunorubicin hydrochloride, 5-fluorouracil, superoxide dismutase (SOD), catalase and hydrogen peroxide (H2O2) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other chemicals were of the highest purity available.

HeLa Cells and Its MDR1-Overexpressing Subline, Hvr100-6 The human cervical carcinoma cell line HeLa (HeLa) was obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). HeLa cells (396–401 passage) were maintained in a culture medium consisting of Dulbecco’s modified Eagle’s medium (D-MEM; Cat. No. 12800–017, Invitrogen Corp., Carlsbad, CA, U.S.A.) supplemented with 10% fetal bovine serum (FBS; Lot. No. AGM7413, HyClone, UT, U.S.A.) and 100 mg/l of kanamycin sulfonate (Invitrogen Corp.). A vinblastine-resistant subline of HeLa, Hvr100-6, was established by stepwise increases of the concentration of vinblastine sulfate in the culture medium.12) Hvr100-6 cells (82–87 passage) were derived and maintained with 100 nm vinblastine sulfate. These cells were seased into culture flasks (Corning Costar Corp., Cambridge, MA, U.S.A.) at a density of 4 and 12 × 105 cells/cm2, respectively, grown in a humidified atmosphere of 5%CO2–95% air at 37 °C, and subcultured every 3 or 4 d with 0.05% trypsin–0.02% EDTA (Invitrogen Corp.).

Effects of ROS, Antioxidants and Anticancer Drugs on the Growth Rate of HeLa and Hvr100-6 Cells The effects of ROS (H2O2 and 3-amino-1,2,4-triazole), antioxidants (baicalein, genistein, quercetin, ascorbic acid, hydroquinone, SOD and catalase) and anticancer drugs (vinblastine sulfate, paclitaxel, doxorubicin hydrochloride, daunorubicin hydrochloride and 5-fluorouracil) on the growth rate of HeLa and Hvr100-6 cells were assessed by the WST-1 (tetrazolium salts) colorimetric assay using a Cell Counting Kit (Dojin Laboratories, Kumamoto, Japan). Cells (1000 cells/well) were seeded on 96-well plates (NunclonTM flasks, Nagle
Nunc International, NY, U.S.A.) in 100 μl of culture medium on Day 0, and the culture medium was changed to that containing a test substance at various concentrations on Day 1. After incubation for 3 d at 37 °C (on Day 4), the culture medium was exchanged for 110 μl of that containing WST-1 reagent solution (10 μl of WST-1 solution and 100 μl of the culture medium), and 3 h later, the absorbance was determined at 450 nm with a reference wavelength of 630 nm using a microplate reader (Sjeia Auto Reader II, Sanko Junyaku Co. Ltd., Tokyo, Japan) according to the manufacturer’s directions. The 50% growth inhibitory concentration (IC50) values in HeLa and Hvr100-6 cells were calculated according to the sigmoid inhibitory effect model as follows using the nonlinear least-squares fitting method (WinNonlin®, ver. 2.1, Pharsight Corp., CA, U.S.A.): \[ E = E_{\text{max}} \times \left[ 1 - \frac{C^\gamma}{C^\gamma + IC_{50}^\gamma} \right] \]

\( E \) and \( E_{\text{max}} \) represent the surviving fraction (% of control) and its maximum, respectively, and \( C \) and \( \gamma \) represent the drug concentration in the medium and the sigmoidicity factor, respectively.

The antioxidants were added simultaneously with a ROS or anticancer drug on Day 1 and growth was assayed similarly. Here, the concentrations of antioxidants were fixed as follows: baicalein (10 μM), genistein (10 μM), quercetin (10 μM), ascorbic acid (250 μM), hydroquinone (6.4 μM), SOD (58 U/ml), or catalase (1300 U/ml). At these concentrations, the antioxidants showed no cytotoxicity themselves (see Results).

**Effects of Antioxidants on MDR1 mRNA Levels in HeLa and Hvr100-6 Cells**

HeLa and Hvr100-6 cells were seeded on dishes at a density of 2 × 105 cells/100 mm dish and 6 × 105 cells/100 mm dishes, respectively, in 10 ml of vinblastine sulfate-free culture medium on Day 0. The culture medium was changed to that containing an antioxidant on Day 1. The concentrations of antioxidants were indicated above. After incubation for 3 d (on Day 4), the cells were scrapped off from subconfluent monolayers and the cell pellet was washed with ice-cold PBS(−) . MDR1 mRNA expression was determined by real time quantitative RT-PCR as described previously.13—15) Total RNA was extracted from tissue samples using an RNeasy Mini kit (QIAGEN, Hilden, Germany) and an RNase-Free DNase Set (QIAGEN) according to the manufacturers’ protocols. The primers and TaqMan probes for MDR1 mRNA were described previously.13—15) In each run of the assay, mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and MDR1 were analyzed in 4-fold serially diluted samples from authentic HeLa, and the mRNA levels of MDR1 were expressed relative to the concentration of GAPDH mRNA. GAPDH was selected as an endogenous RNA control to normalize for differences in the amount of total RNA.

**Statistical Analysis**

Values are given as the means±S.D. Multiple comparison was performed by one-way analysis of variance (ANOVA) followed by Dunnet’s multiple comparison, providing the variance of the groups was similar. p values of less than 0.05 (two-tailed) were considered significant.

**RESULTS**

**Effects of ROS on Growth Rate of HeLa and Hvr100-6 Cells**

Figure 1 shows the growth inhibitory curves of HeLa and Hvr100-6 cells for H2O2. For HeLa cells, H2O2 enhanced cell growth at about 0.2—20 nm, while H2O2 showed cytotoxicity at higher concentrations in a concentration-dependent manner (Fig. 1A). The catalase inhibitor 3-amino-1,2,4-triazole had no such effect (data not shown). The growth enhancement caused by H2O2 in HeLa cells was suppressed by the addition of 58 U/ml SOD or 1300 U/ml catalase. Each column represents the mean±S.D. of 3—4 experiments. † p<0.05: significantly different compared with the first control. * p<0.05: significantly different compared with the second control.
shows the effects of antioxidants on the growth enhancement of HeLa cells by H₂O₂. The enhancement at 1.0 nM H₂O₂ was suppressed by baicalein (10 μM), genistein (10 μM), hydroquinone (6.4 μM), SOD (58 U/ml), or catalase (1300 U/ml). In HeLa cells, the IC₅₀ values of these antioxidants were 49.0±4.69 μM, 33.5±2.37 μM, 209±13.3 μM, 998±69.9 μM, 12.8±2.78 μM, 705±189 U/ml and more than 2000 U/ml, respectively, while in Hvr100-6 cells, they were 49.5±7.64 μM, 41.6±6.12 μM, 209±35.3 μM, 687±104 μM, 12.1±2.35 μM, 385±142 U/ml and more than 2000 U/ml. Thus, the suppressive effects of antioxidants on growth enhancement of HeLa cells by 1.0 nM H₂O₂ were not due to cytotoxicity of the antioxidants themselves.

Effects of Doxorubicin and Daunorubicin on Growth Rate of HeLa and Hvr100-6 Cells
Figure 3 shows the growth inhibitory curves of HeLa and Hvr100-6 cells for doxorubicin and daunorubicin. For HeLa cells, doxorubicin and daunorubicin enhanced cell growth at a concentration lower than their IC₅₀ values in a concentration-dependent manner (Figs. 3A, C). This was not observed for vinblastine, paclitaxel, or 5-fluorouracil (data not shown). The growth enhancement caused by doxorubicin and daunorubicin in HeLa cells was suppressed by the addition of 58 U/ml SOD or 1300 U/ml catalase to the culture medium (Figs. 3A, C). The growth enhancement was not observed in Hvr100-6 cells (Figs. 3B, D). Figure 4 shows the effects of antioxidants on the growth enhancement by daunorubicin. The enhancement at 0.25 nM was suppressed by 10 μM baicalein, 10 μM genistein, 250 μM ascorbic acid, 58 U/ml SOD, or 1300 U/ml catalase.

Effects of Antioxidants on MDR1 mRNA Levels in HeLa and Hvr100-6 Cells
MDR1 mRNA levels (± S.D.; N=4) were 0.89±0.19 and 742.42±70.30 in HeLa and Hvr100-6 cells, respectively. These were not altered by either 10 μM baicalein, 10 μM genistein, 10 μM quercetin, 250 μM ascorbic acid, 6.4 μM hydroquinone, 58 U/ml SOD, or 1300 U/ml catalase in HeLa or Hvr100-6 cells (data not shown). The cytotoxicity of vinblastine, paclitaxel, daunorubicin, daunorubicin and 5-fluorouracil in HeLa and Hvr100-6 cells were not altered by addition of these antioxidants (data not shown).

DISCUSSION
Herein, it was confirmed that the growth of HeLa cells was enhanced by H₂O₂, a typical ROS at concentrations lower than their IC₅₀ values in a concentration-dependent manner, and the growth enhancement was suppressed by antioxidants. Doxorubicin and daunorubicin also enhanced the growth of HeLa cells and this was suppressed by antioxidants, being consistent with the fact that doxorubicin and daunorubicin generate ROS.5,6) However, the growth enhancement by H₂O₂ or doxorubicin and daunorubicin was not observed in the MDR1-overexpressing subline, Hvr100-6 cells. Hvr100-6 cells were established through stepwise increases of the vinblastine concentration, and the expression levels of a large number of proteins should be altered including MDR1. Thus, it is difficult to explain why the growth enhancement by H₂O₂ or doxorubicin and daunorubicin was not observed in Hvr100-6 cells. Expression levels of MDR1 mRNA were not altered during 3 days incubation and/or addition of antioxidants, and further experiments should be addressed using MDR1-transfecting cells.

Recently, Ziemann et al. have indicated that the addition of H₂O₂ or catalase inhibitor induced the expression of MDR1 and its mRNA, and antioxidants markedly suppressed this in primary rat hepatocyte cultures.10) This is contradictory to our observation, suggesting that the transcriptional factors necessary for MDR1 induction by H₂O₂ were not expressed in HeLa cells, or that the MDR1 induction system in rat hepatocytes was different from that in human cancer cells. Any-
way, the possibility of using antioxidants to reverse multidrug resistance was still unclear.

REFERENCES AND NOTES

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