Direct Measurement of Doxorubicin Concentration in the Intact, Living Single Cancer Cell during Hyperthermia

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BACKGROUND. It is well known that the effect of doxorubicin on cancer cells is enhanced by hyperthermia. The mechanism of this phenomenon is not fully understood.

METHODS. Two esophageal squamous cell carcinoma cell lines, TE-2 and TE-6, were used; these cell lines have different sensitivities for doxorubicin. The cells were exposed to 1 μg/mL of doxorubicin for 30 minutes. With a confocal laser scanning microscope and a transparent warming plate, doxorubicin concentration was measured continuously in the intact, living single cancer cells, and the two-dimensional distribution of the drug during hyperthermia (43 °C) was analyzed.

RESULTS. A doxorubicin sensitivity difference was confirmed between TE-2 and TE-6 cells by colonogenic assay ($P < 0.05$). Hyperthermia increased the sensitivity of both cell lines to the drug ($P < 0.05$) and eliminated the sensitivity difference. Doxorubicin accumulated in the nuclei in both cell lines 30 minutes after exposure to the drug in a time-dependent manner ($P < 0.05$). Without hyperthermia, the doxorubicin concentration in the nuclei of the TE-2 cells (4.8 ± 0.3 μg/mL) was higher than in the nuclei of the TE-6 cells (2.3 ± 0.5 μg/mL) ($P < 0.05$). With hyperthermia, there was no significant difference in doxorubicin concentration between the nuclei of the TE-2 cells (20.8 ± 1.3 μg/mL) and the nuclei of the TE-6 cells (16.5 ± 3.9 μg/mL).

CONCLUSIONS. Hyperthermia increased the uptake of doxorubicin in the nuclei of cancer cells. Thus, the authors concluded that hyperthermia increases the cells' sensitivity to the drug. ***Cancer* 1997; 79:214–9.** © *1997 American Cancer Society.*

KEYWORDS: hyperthermia, doxorubicin, squamous cell carcinoma, confocal laser scanning microscope, imaging.

Hyperthermia is a well-established therapy for many kinds of cancer. It is sometimes combined with chemotherapy[1–5] and/or radiation.[6,7] Many investigators have reported that hyperthermia enhanced the effect of chemotherapy in clinical[1–5] and laboratory studies.[8–12] Even in doxorubicin-resistant cells, hyperthermia enhanced the effect of doxorubicin.[13] Although some studies that used flow cytometry reported that this enhancement was due to the doxorubicin concentration increase in the cells,[8,13] the subcellular distribution of the drug during hyperthermia has not been demonstrated. To further our understanding of the mechanism of this enhancement effect of hyperthermia, we attempted to determine the subcellular distribution of doxorubicin during hyperthermia. Using a fluoroimaging technique with a confocal laser scanning microscope (CLSM) and a transparent warming plate, we measured the doxorubicin concentration continuously in intact, living single cancer cells, and we examined the two-

dimensional distribution of the drug in the cells during hyperthermia. We found that the hyperthermia dramatically increased the doxorubicin concentration in the nuclei of the cancer cells.

MATERIALS AND METHODS

Cell Culture

Two esophageal squamous cell carcinoma cell lines, TE-2 and TE-6, were used. These cell lines were kindly provided by Dr. T. Nishihira, Tohoku University School of Medicine, Sendai, Japan. Cells were grown to the point of subconfluence in RPMI-1640 medium (Nissui, Tokyo, Japan), supplemented with 10% fetal calf serum (GIBCO, NY) and the antibiotics penicillin G (100 units/mL) and streptomycin (100 μg/mL), in plastic flasks (for colonogenic assay) or plastic dishes (for doxorubicin concentration measurement) at 37 °C with 5% CO_2 in the surrounding air.

Colonogenic Assay

To analyze the sensitivity of the cancer cells to doxorubicin with and without hyperthermia, the colonogenic assay was carried out on TE-2 and TE-6 cells in flasks. Doxorubicin (Adriamycin, Adria Laboratories, Columbus, OH; a gift from Kyowa Hakkou, Tokyo, Japan) was added to the cells in the flask at different final concentrations (0.2 and 0.5 μg/mL). The flask was incubated for 30 minutes in a water bath at 37 °C (for normothermia groups) or 43 °C (for hyperthermia groups). Immediately after incubation, cells were detached with a short exposure to trypsin and ethylenediamine tetraacetic acid in phosphate-buffered saline (PBS) lacking calcium $(Ca)^{2+}$ and magnesium $(Mg)^{2+}$. One thousand cells were then subcultured in 6-well dishes and cultured in the same medium for 14 days, at the end of which the number of colonies was counted. The number of colonies was divided by the number of the untreated controls. The survival fraction was defined as (plating efficiency of experimental group)/(plating efficiency of untreated control).

Experimental System for Doxorubicin Concentration Measurement

The confocal imaging system used was a LSM 410 scanning assembly (Zeiss, Germany) incorporating a 100 mW argon ion laser and coupled to a Axioverd 135 fluorescence microscope (Zeiss). The cell culture dishes were heated with a transparent warming plate, the Microwarm Plate™ (Olympus, Tokyo, Japan), which was placed on the stage of the microscope. The temperature of the medium and the bottoms of the dishes were continuously measured with a thermometer (HEH-TM4, OMRON, Osaka, Japan). The accuracy of the temperature control of the warming plate was always within ±0.5 °C. The

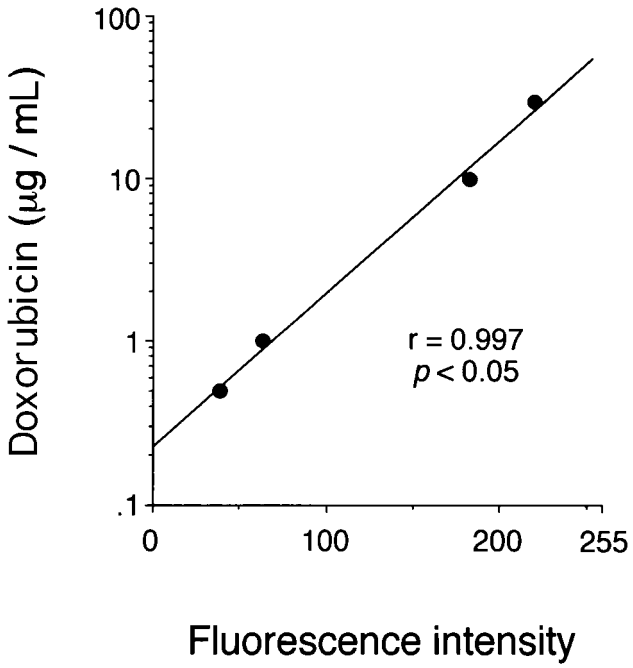

FIGURE 1. A calibration line is shown. The known concentration of doxorubicin solution was visualized by a confocal laser scanning microscope, and the fluorescence intensity was measured by a computer image analyzing system. The fluorescence intensity of doxorubicin was always highly correlated with the log-doxorubicin concentration.

fluorescence images were recorded on a hard disk through the CLSM at the excitation laser wave length 488 nm. The objective lens was used at magnification ×40 for image analysis. After the experiment, the fluorescence intensity of the recorded images was analyzed by personal computer and image-analyzing software (NIH Image, version 1.55) and expressed as 256 gray levels. Before each cell experiment, the fluorescence intensity of the known concentration of doxorubicin solution (0.5, 1, 5, and 10 μg/mL) was measured in a plastic cell culture dish on the warming plate. The fluorescence intensity of the different concentrations of doxorubicin was plotted against the doxorubicin concentration (calibration line) (Fig. 1). The correlation between doxorubicin concentration and fluorescence intensity was a log-linear relation. The correlation between the log-doxorubicin concentration and fluorescence intensity was always significant. Therefore, we were able to calculate the concentration of doxorubicin in the recorded images from the calibration line that we had made before each cell experiment using known concentrations of the drug.

Experimental Groups and Doxorubicin Concentration in the Intact, Living Single Cells

We made four experimental groups: two normothermia groups and two hyperthermia groups. The normo-

thermia groups were (1) the TE-2 (37 °C) group, in which TE-2 cells were exposed to 1 mg/mL of doxorubicin for 30 minutes at 37 °C, and (2) the TE-6 (37 °C) group, in which TE-6 cells were exposed to 1 μg/mL of doxorubicin for 30 minutes at 37 °C. The hyperthermia groups were (1) the TE-2 (43 °C) group, in which TE-2 cells were exposed to 1 μg/mL of doxorubicin for 30 minutes at 43 °C, and (2) the TE-6 (43 °C) group, in which TE-6 cells were exposed to 1 μg/mL of doxorubicin for 30 minutes at 43 °C.

The subconfluent cells on plastic dishes were placed on the warming plate and warmed at 37 °C. The cells were washed twice with PBS and the medium was replaced with prewarmed Hanks' solution. Immediately after 1 μg/mL of doxorubicin (final concentration) was added to the dish, the dish was warmed to 43 °C in the hyperthermia groups, whereas the temperature setting was maintained at 37 °C in the normothermia groups. The doxorubicin fluorescence images were recorded on a hard disk every 5 minutes for 30 minutes. The phase contrast image of the cancer cells was recorded before each experiment. The nucleus, cytoplasm, and medium area were selected in this phase contrast image. The average fluorescence intensity in the selected area was then calculated with the image-analyzing software. The doxorubicin concentrations of the nucleus, cytoplasm, and medium were calculated from the calibration line that was made before each experiment.

Statistical Methods

Values were expressed as mean ± standard error. The significance of the differences among groups was assessed by one-way analysis of variance with Dunnett's multiple comparison test. Differences among groups were considered significant when $P < 0.05$.

RESULTS

Colonogenic assay was used to determine (1) the sensitivity of both TE-2 and TE-6 cell lines to doxorubicin and (2) the effect of hyperthermia on the sensitivity to the drug. Without hyperthermia, the sensitivity of the TE-2 cells to doxorubicin was higher than that of the TE-6 cells ($P < 0.05$) (Fig. 2). However, with hyperthermia, there was no significant difference between the sensitivity of TE-2 to doxorubicin and that of TE-6. Furthermore, hyperthermia significantly increased the sensitivity of both cancer cell lines to the drug ($P < 0.05$).

Figure 3 shows a typical two-dimensional distribution of doxorubicin fluorescence in an intact, living single TE-2 cancer cell with hyperthermia. In all series of experiments, doxorubicin accumulated in the nuclei of the cancer cells (Fig. 3B). The doxorubicin fluorescence intensity in the perinuclear area of the cytoplasm was also higher than that in the other areas of the cytoplasm and in the medium (Fig. 3C). After the experiment, we calculated the doxorubicin concentrations of the nuclei and the cytoplasm of single cancer cells using a calibration line for each experiment. Without hyperthermia, the doxorubicin concentration in the nuclei of TE-2 cells was higher than that of the TE-6 cells ($P < 0.05$) (Fig. 4A). Hyperthermia increased the doxorubicin concentration in the nuclei of both cell lines in a time-dependent manner (Fig. 4A,B). Furthermore, there was no significant difference in doxorubicin concentration between the nuclei of the TE-2 cells and those of the TE-6 cells with hyperthermia at 15, 20, 25, and 30 minutes (Fig. 4B). The cytoplasmic doxorubicin concentration in the TE-2 cells was always higher than in the TE-6 cytoplasm ($P < 0.05$) (Fig. 4C,D).

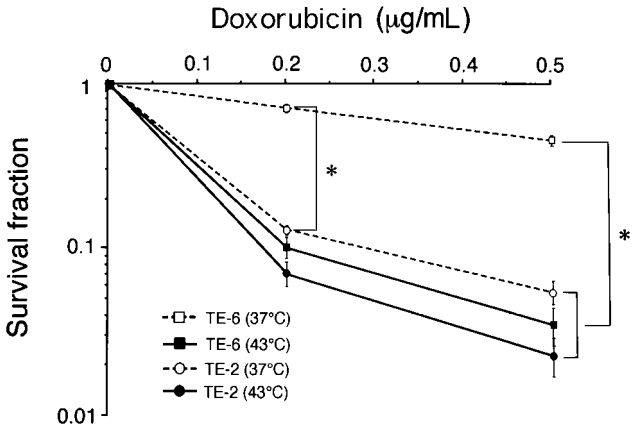

FIGURE 2. The enhancement effect of hyperthermia on the sensitivity of the cancer cells to doxorubicin is shown. Values are mean ± standard error (n = 4 in each group). The four groups of cultured squamous cell carcinoma cell lines (TE-2 and TE-6) used in the study are represented. *: $P < 0.05$. There was no statistical difference between the survival fraction of TE-6 and that of TE-2 with hyperthermia alone. The survival fraction of the TE-6 (37 °C) group was higher than that of the TE-6 (43 °C), TE-2 (37 °C), TE-2 (43 °C) groups. There was no significant difference among the TE-6 (43 °C) group, TE-2 (37 °C) group, and TE-2 (43 °C) groups.

DISCUSSION

In this study, we demonstrated the two-dimensional distribution of doxorubicin in cancer cells and continuously measured the concentration of the drug in single cancer cells during hyperthermia by means of a fluoroimaging technique with a CLSM during the hyperthermia. We found that the hyperthermia dramatically increased doxorubicin uptake in the nuclei of

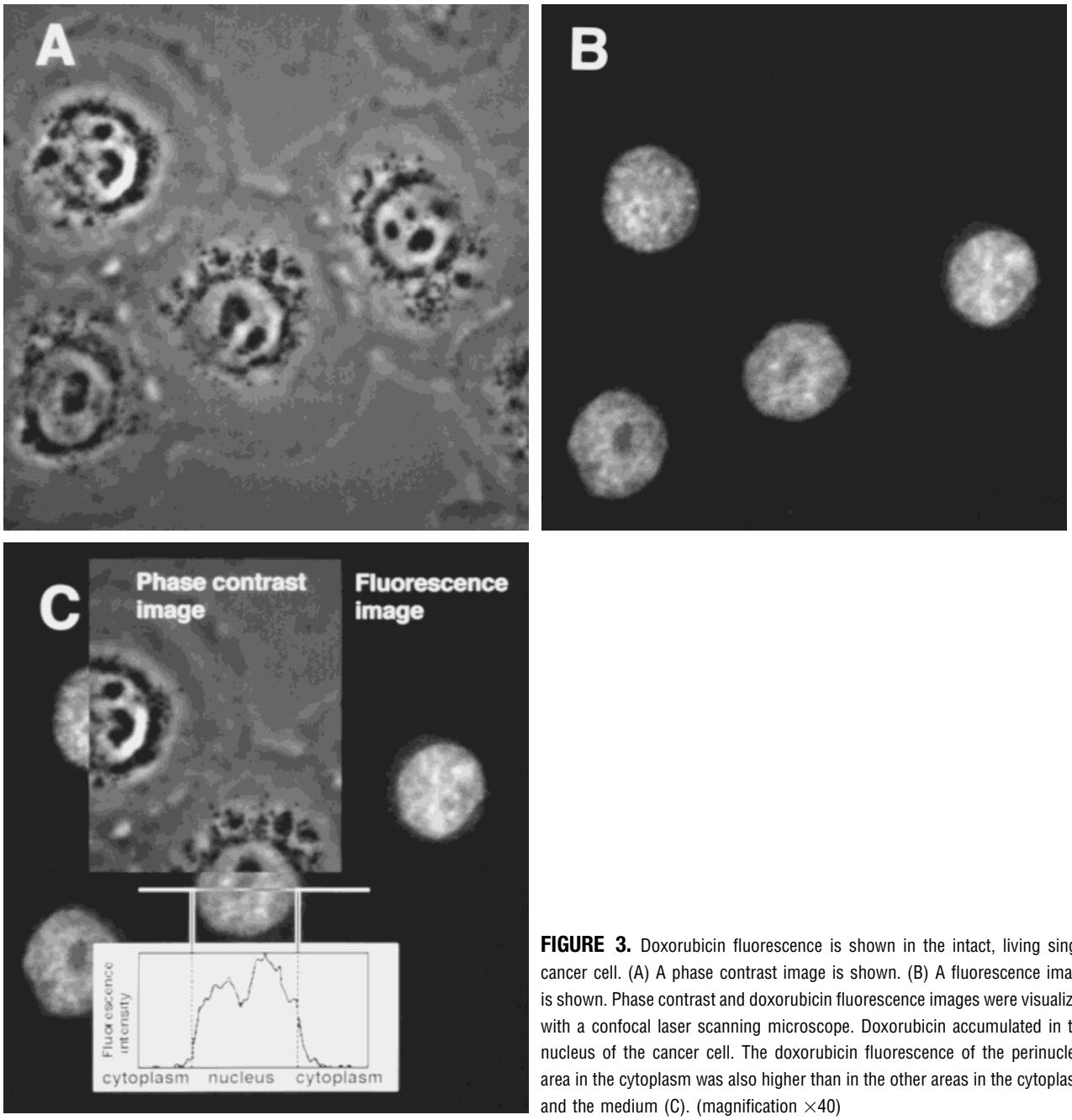

FIGURE 3. Doxorubicin fluorescence is shown in the intact, living single cancer cell. (A) A phase contrast image is shown. (B) A fluorescence image is shown. Phase contrast and doxorubicin fluorescence images were visualized with a confocal laser scanning microscope. Doxorubicin accumulated in the nucleus of the cancer cell. The doxorubicin fluorescence of the perinuclear area in the cytoplasm was also higher than in the other areas in the cytoplasm and the medium (C). (magnification $\times 40$)

cancer cells and that the hyperthermia increased the sensitivity of the cancer cells to the drug.

The rejection of out-of-focus background is the most powerful advantage of using CLSM for fluoroimaging.[14] We were thereby able to measure the fluorescence intensity correctly without any influence from out-of-focus fluorescent material. The second advantage of CLSM is that its image resolution is higher than that of fluorescence light microscopy.[14] Although several investigators have used CLSM to demonstrate the distribution of doxorubicin in single cancer cells, they did not measure the concentration of the drug, only its fluorescence intensity.[15–17] We made the best use of these advantages of CLSM. After we measured the fluorescence intensity of known concentrations of doxorubicin and made a calibration line, we calculated the doxorubicin concentration of the cancer cell from the drug's fluorescence intensity in the cell using the calibration line. Furthermore, with the addition of the warming plate to the CLSM system,

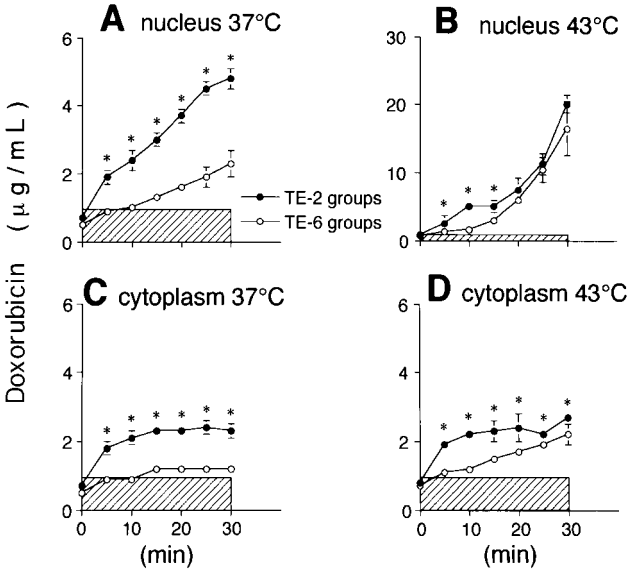

FIGURE 4. The effect of hyperthermia on doxorubicin concentration in intact, living single cancer cells is shown. The concentrations in the nucleus and the cytoplasm were measured by means of a fluoroimaging technique with a confocal laser scanning microscope. Nine samples in each group were examined. Values are mean ± standard error TE-2 and TE-6 groups represent the groups of cultured esophageal squamous cell carcinoma cell lines used in the study. Hatched squared area: doxorubicin concentration in medium (1 μg/mL); *: $P < 0.05$ (TE-2 vs. TE-6). Note the scale difference between graph B and other graphs (A, C, D). Hyperthermia dramatically increased the doxorubicin concentration in the nuclei of both cell lines. The doxorubicin concentrations in the nuclei of both cell lines with hyperthermia at 15, 20, 25, and 30 minutes were higher than those without hyperthermia ($P < 0.05$). The doxorubicin concentration difference in the cell nuclei between TE-2 and TE-6 cells without hyperthermia disappeared with hyperthermia. There was no significant difference between the concentration in the cytoplasm of TE-2 cells with hyperthermia and that without hyperthermia. The concentrations in the cytoplasm of the TE-6 cells with hyperthermia at 15, 20, 25, and 30 minutes were higher than those without hyperthermia ($P < 0.05$).

we were able to measure the doxorubicin concentration of the single cancer cell continuously during hyperthermia and were thus able to demonstrate the two-dimensional distribution of the drug in single cancer cells.

Doxorubicin was accumulated in the nuclei of the cancer cells in all groups. These results were consistent with previous studies, which reported that doxorubicin was localized inside the nucleus.[15,17] It is well known that doxorubicin intercalates with DNA.[18] Furthermore, the differences in distribution of doxorubicin between the nucleus and the cytoplasma may explain the selective reduction of intranuclear glutathione by doxorubicin.[19] Therefore, doxorubicin concentration in the nucleus may increase until DNA is saturated with it. In our present experiments, the concentration of the drug in the nucleus was higher than that in the medium and continuously increased over time. The doxorubicin concentration in the cytoplasm was also higher than in the medium (Fig. 4C,D), especially in the perinuclear area (Fig. 3C). Doxorubicin may bind to other subcellular organelles, such as cytoskeleton[20,21] and membranes.[22] However, it has been reported that doxorubicin is located in the cytoplasm but not in the nucleus in doxorubicin-resistant cells[15] as well as cells that are resistant to multiple drugs.[16] Therefore, we hypothesized that doxorubicin concentration in the nucleus may be related to the cell's sensitivity to the drug. We found that the doxorubicin concentration of TE-2 cells without hyperthermia (which had a lower sensitivity to doxorubicin compared to TE-2 cells with hyperthermia) and the doxorubicin concentration of TE-6 cells with and without hyperthermia were lower than that of TE-2 cells with hyperthermia and lower than that of TE-6 cells with and without hyperthermia. Although we observed that hyperthermia dramatically increased both the sensitivity to doxorubicin and the concentration of the drug in the nuclei of cancer cells, the mechanism of the increase in concentration is still unknown. We speculate that hyperthermia may increase the nucleus membrane permeability for doxorubicin, thus allowing the doxorubicin concentration to increase in the nucleus.

The methods used in this study to demonstrate the two-dimensional distribution of doxorubicin in single cancer cells and measure the drug's concentration during hyperthermia should also be useful for the study of cancer cells that are resistant to multiple drugs. The dramatic change that we observed in doxorubicin uptake in the nuclei of cancer cells may contribute to the use of hyperthermia to enhance doxorubicin sensitivity in these cells.

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