Chronic effect of doxorubicin on vascular endothelium assessed by organ culture study

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Abstract

We have attempted to determine the chronic effects of doxorubicin, a commonly used anticancer agent, on vascular endothelium using an organ culture system. In rabbit mesenteric arteries treated with 0.3 μM doxorubicin for 7 days, rounding and concentrated nuclei and TUNEL-positive staining were observed in endothelial cells, indicating DNA damage and the induction of apoptosis. However, the endothelium-dependent relaxation induced by substance P and the expression of mRNA encoding endothelial NO synthase (eNOS) did not differ from those in control arteries. In arteries treated with a higher concentration (1 μM) of doxorubicin, apoptosis and damage to nuclei occurred in the endothelial cells at the third day of treatment, and the detachment and excoriation of endothelium from the tunica interna of the vascular wall were also observed. The impairment of endothelium-dependent relaxation was observed at the fifth day of the treatment with 1 μM doxorubicin. Additionally, apoptotic change in the smooth muscle layer was observed at this concentration of doxorubicin. Apoptotic phenomena were further confirmed by DNA fragmentation using isolated bovine aortic endothelial cells (BAECs) and A7r5 vascular smooth muscle cells, and it was revealed that BAECs are more sensitive than A7r5 to the apoptotic effect of doxorubicin. These results suggest that chronic treatment with doxorubicin at therapeutic concentrations induces apoptosis and excoriation of endothelial cells, which diminishes endothelium-dependent relaxation. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Doxorubicin; Vascular organ culture; Endothelium-dependent relaxation; Apoptosis

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Introduction

Doxorubicin is one of the most widely used broad spectrum anticancer agents [1, 2]. However, its long-term administration in humans is limited because of the development of a dose-related cardiomyopathy [3, 4]. Laboratory studies have attempted to elucidate the mechanism of doxorubicin-induced cytotoxicity using various cell lines, and suggested that doxorubicin can intercalate with DNA resulting in changes in DNA functions including the inhibition of DNA and RNA synthesis [5], the occurrence of strand breaks, and the induction of apoptosis [6]. However, its role in the mechanism of the heart failure is still unclear.

An in vivo study indicated that doxorubicin induced apoptosis in renal tubules and epithelium of the intestinal mucosa, but not in cardiac myocytes, suggesting that multiple cytotoxic mechanisms are involved in doxorubicin-induced cytotoxicity [7]. Among the doxorubicin-toxicity, dysfunction of circulatory system including hypotension or compensative hypertension is serious, because it is often observed in clinical situations such as repetitive doxorubicin treatment [8, 9, 10, 11]. During the course of i.v. injection of doxorubicin, vasculature is exposed to high levels of doxorubicin over a long period of time. Previously, doxorubicin above the therapeutic concentration has been reported to induce acute vasoconstriction by releasing Ca$^{2+}$ from its intercellular storage site [12]. However, the long-term effect of doxorubicin at therapeutic concentration on vasculature had not been clarified. We determined that doxorubicin has serious toxic effect on vascular smooth muscle after repetitive and long-term treatment [13]. Although vascular endothelium is considered to play important roles in the regulation of vascular tone and protection of vascular smooth muscle, the long-term toxicity of doxorubicin on vascular endothelium has not been elucidated thus far.

In the present report, we investigated the chronic effects of doxorubicin on morphological and functional changes of vascular endothelium using an organ culture method. Our results indicate that endothelium is more sensitive than smooth muscle cells to chronic treatment with therapeutic concentrations of doxorubicin, which induces apoptosis and impairs the vasodilator effect.

Materials and methods

Tissue preparation and organ culture procedure

Male Japanese White rabbits (2–3 kg) were euthanized by stunning and exsanguination. Organ culture procedure was performed as described previously [13]. Briefly, main branches of the superior mesenteric arteries were isolated. After removing fat and adventitia in sterile Hanks’ balanced salt solution, each artery was cut into rings (approximately 2 mm wide). Arterial rings were then placed in 2 ml Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 1% penicillin-streptomycin. Some rings were treated with similar DMEM solutions with doxorubicin (0.3–1 μM). The muscle rings were maintained at 37 °C in an atmosphere of 95% air and 5% CO$_2$ for 3, 5, or 7 days. The medium was changed in the presence or absence of doxorubicin every 2 days until the experiments were initiated. This interval (7 days) was selected on the basis of our previous experiments in which vascular morphology and function were well maintained in the rabbit mesenteric arteries cultured in the serum-free DMEM for 7 days [14], and based on other reports which indicated that the phar-
Macological and toxic effects of therapeutic doses of doxorubicin are maximal between 1 and 2 weeks after administration of the drug [15]. Animal care and treatment were conducted in conformity with institutional guidelines of The University of Tokyo.

**Morphological examinations**

The tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin. The 4 μm-thick-sections were stained with hematoxylin and eosin (HE). The slides were examined by light microscope. For the detection of apoptosis in situ, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay was performed using a commercially available kit (In Situ Apoptosis Detection Kit-POD, Boehringer Mannheim, Japan). Briefly, deparaffinized sections were first treated with proteinase K (20 μg/ml) and then blocked for endogenous peroxidase activity using 3% H2O2. End-labeling with TdT (0.3 U/μg) conjugated with FITC-labeled 16-UTP (2 μM) was performed for 1 hour at 37°C in a humidifying chamber. The slides were examined by laser scanning microscope. TUNEL-positive apoptotic nuclei were identified by the presence of green staining.

**Bovine aortic endothelial cell and A7r5 smooth muscle cell culture**

Bovine aorta was obtained from a nearby slaughterhouse and transported in sterilized phosphate buffered saline at 4°C. Bovine aortic endothelial cells (BAECs) were collected by 0.05% trypsin-EDTA digestion and cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin. Rat aortic vascular smooth muscle cell line, A7r5, was purchased from Dainippon Pharmaceutical, Japan. Passage 8 of BAECs showing cobblestone-like morphology and passage 20 of A7r5 were used for experiments. Cells were cultured for 24 hours in serum-free DMEM prior to doxorubicin treatment.

**Analysis of DNA fragmentation**

Cells were incubated in lysis buffer (trisaminomethane (Tris)-HCl 10.0 mM (pH 7.4), NaCl 150.0 mM, EDTA 10.0 mM, and 0.1% sodium dodecylsulfate) with 100 μg/ml proteinase K at 55°C for 100 min. DNA was extracted once using equal volumes of phenol (PhOH). The upper aqueous phase containing DNA was extracted with equal volumes of PhOH/chloroform (CHCl3) (1:1) and precipitated with ethanol. Finally, the DNA pellet was resuspended in 10 mM Tris-HCl (pH 7.4)-1 mM EDTA (TE) buffer. DNA concentration was measured using a spectrophotometer at 260 nm. Four μg of each DNA was loaded onto 2.0% agarose gel containing 0.1% ethidium bromide. Electrophoresis was performed at 100 V for 40 min and detectable fluorescent bands were visualized using a UV-transilluminator.

**Semiquantitative RT-PCR**

Total RNA was extracted from the arterial rings and BAECs using the acid guanidinium thiocyanate-phenol-chloroform method employing the TRIzol reagent. The concentration of total RNA was adjusted to 1 μg/μl with RNase-free distilled water. RT-PCR was performed as described previously [16]. Briefly, the first strand of cDNA was synthesized using random 9 mers RT-primer and AMV Reverse Transcriptase XL at 30°C for 10 min, 55°C for 30 min, 99°C for 5 min, and 4°C for 5 min. PCR amplification using synthetic gene-specific primers
for endothelial NO synthase and GAPDH. PCR amplification was performed using Taq DNA polymerase (Ampli Taq Gold). The oligonucleotide primers for endothelial NO synthase and GAPDH were designed as described previously [17]. The forward primers and reverse primers for endothelial NO synthase and GAPDH were designed as follows: ATA GAA TTC ACC AGC ACC TTT GGG AAT GGC GAT (forward primer for endothelial NO synthase), ATA GAA TTC GGA TTT ACT GTG GTC TGT GCT GGA CTC CTT (reverse primer for endothelial NO synthase), TCC CTC AAG ATT GTC AGC AA (forward primer for GAPDH), and AGA TCC ACA ACG GAT ACA TT (reverse primer for GAPDH). After denaturation at 95°C for 10 min, 27–39 cycles of amplification at 94°C for 0.4 min, 55°C for 1 min, and 72°C for 1.5 min were performed using a thermal cycler (Takara PCR Thermal Cycler MP, Takara Biomedicals, Japan). PCR-products were electrophoresed onto 2% agarose gel containing 0.1% ethidium bromide. The possibility of containing DNA was excluded by a PCR with total RNA without the reverse transcription step. Electrophoresis was performed at 100 V for 40 min and detectable fluorescent bands were visualized using a UV-transilluminator. The densitometric intensity at 30 cycles was quantified using NIH Image. The results were expressed as the ratio of the optical density to GAPDH.

Measurement of muscle tension

After the incubation, the arterial rings were placed in normal physiological salt solution (PSS) with the following composition: NaCl 136.9 mM, KCl 5.4 mM, CaCl₂ 1.5 mM, MgCl₂ 1.0 mM, NaHCO₃ 23.8 mM, and glucose 5.5 mM. Ethylenediaminetetraacetic acid (EDTA, 1µM) was also added to remove contaminating heavy metal ions which the catalyze oxidation of organic chemicals. The high-K⁺ solution was prepared by replacing NaCl with equimolar KCl. The PSS was saturated with a 95% O₂-5% CO₂ mixture at 37°C and pH 7.4. Muscle tension was recorded isometrically with a force displacement transducer (Orientic, Japan). Data were shown as a percent of relaxation of the steady-state preconstriction.

Chemicals

The chemicals used were as follows: doxorubicin, proteinase K, substance P (Sigma, USA), random 9 mers RT-primer (Takara Biomedicals, Japan), AMV Reverse Transcriptase XL (Life Science, USA), Hanks’ balanced salt solution, penicillin-streptomycin, TRizol reagent, penicillin-streptomycin, ethidium bromide solution (GIBCO BRL, USA), DMEM (Nissui Pharmaceutical, Japan), and Taq DNA polymerase (Roche, USA).

Statistical analysis

Results of experiments are expressed as means ± S.E.M. One-way ANOVA (Bonferroni’s test) was used for statistical analysis of results and P < 0.05 was taken as significant.

Results

Morphological examination

Figure 1 shows the HE staining section of the vascular tissue after up to 7 days of treatment without or with 0.3 or 1 µM doxorubicin. The endothelial cells of the arteries cultured...
without doxorubicin for 7 days (control arteries) attached tightly to the tunica interna (n=5, Fig. 1 A). In the arteries cultured with doxorubicin 0.3 μM for 3 or 5 days, conspicuous changes of endothelial morphology were not observed when compared with the control arteries (n=5, each, Figs. 1 B and C). In the arteries treated with 0.3 μM doxorubicin for 7 days, in contrast, the excoriation of endothelial cells from the tunica interna were observed in parts, and apoptosis-like concentrated nuclei were often observed. However, the morphology of smooth muscle cells was not changed compared with that of the control arteries (n=8, Fig. 1 D).

In the arteries treated with 1 μM doxorubicin for 3 days, partial excoriation from the tunica interna and concentrated nuclei were observed in the endothelial cells (n=6, Fig. 1 E). In the arteries treated with 1 μM doxorubicin for 5 days, many endothelial cells were excoriated and had dropped off, and typical apoptotic endothelial cells were often observed with concentrated and rounding nuclei (n=7, Fig. 1 F). Additionally, most endothelial cells had dropped off in the arteries treated with 1 μM doxorubicin for 7 days (n=6, Fig. 1 G). In the smooth muscle layer of the tunica media, typical apoptotic phenomenon such as concentrated nuclei was observed at the fifth or seventh day of the treatment with 1 μM doxorubicin.

To further examine the effects of doxorubicin on endothelium, we performed TUNEL assay which can label apoptosis-induced DNA strand breaks. In the control arteries were no TUNEL-positive endothelial cells (n=5, Fig. 2 A). In the arteries treated with 0.3 μM doxorubicin for 3 or 5 days, a few TUNEL-positive endothelial cells were visible (n=6, each, Figs. 2 B and C). In the arteries treated with 0.3 μM doxorubicin for 7 days, many TUNEL-positive endothelial cells were observed (n=8, Fig. 2 D). In the arteries treated with 1 μM doxorubicin for 3, 5, and 7 days, in contrast, TUNEL-positive endothelial cells were often observed (n=7, each, Figs. 2 E, F, and G). In the arteries treated for 5 and 7 days, TUNEL-positive smooth muscle cells were also observed.
Analysis of DNA fragmentation in culture cells

To confirm the results of the TUNEL assay, we detected the internucleosomal DNA fragmentation in cultured endothelial and smooth muscle cells using agarose gel electrophoresis. In the DNA preparations extracted from doxorubicin non-treated (control) BAECs and A7r5, no DNA cleavage was visualized (n=4, Fig. 3 A, B and C). In the DNA preparations extracted from BAECs treated with 0.3 or 1 μM doxorubicin for 12 h, however, DNA cleavage bands with a characteristic pattern of internucleosomal ladder were observed (n=4, Fig. 3 A). Such DNA fragmentation was not obvious in the DNA samples extracted from A7r5 treated with 0.3 or 1 μM doxorubicin for 12 h (n=4, Fig. 3 B). However, the treatment with 1 μM doxorubicin for 48 h induced apparent internucleosomal DNA fragmentation in A7r5 (n=4, Fig. 3 C).

Endothelium-dependent relaxation

In the preliminary experiments, we examined the acute effect of doxorubicin on smooth muscle contractility and endothelium-dependent vasodilation in freshly isolated arteries and found that doxorubicin induced neither contraction nor relaxation at a concentration of 0.3–10 μM. In the arteries cultured without doxorubicin for 3, 5, or 7 days (control arteries), 100 nM substance P inhibited the 35 mM K⁺-induced submaximal contraction by 63.2±5.9% (n=17), 53.2±3.2% (n=21), and 54.1±2.7% (n=25), respectively (Fig. 4). In the arteries treated with 0.3 μM doxorubicin for 3, 5, and 7 days, endothelium-dependent relaxation elicited by substance P (100 nM) did not change from that in the control arteries (57.6±2.9%; n=22, 48.3±3.1%; n=17, and 44.9±3.1%; n=23, respectively). In the arteries treated with 1 μM doxorubicin for 3 days, the relaxation did not change from that in control arteries (58.3±5.8%; n=15). On the other hand, in the arteries treated with 1 μM doxorubicin for 5
or 7 days, relaxations significantly decreased to $12.2 \pm 2.0\%$ ($n=23$) and $12.8 \pm 1.9\%$ ($n=18$), respectively. In all of the arteries, substance P was ineffective when endothelium was removed, whereas the treatment with indomethacin ($10 \mu M$, 30 min) had no effect on substance P-induced relaxation ($n=5$, data not shown). The substance P ($100 \text{nM}$)-induced relaxation was abolished by the treatment with L-NMMA ($200 \mu M$, 30 min) (data not shown).

Expression of endothelial NO synthase mRNA

We examined the effects of doxorubicin on the mRNA transcription steps of endothelial NO synthase using semiquantitative RT-PCR. In the preliminary experiments, we confirmed that organ culture procedure (7 days) and doxorubicin treatment (7 days for tissues, 12 h for cells) did not affect the expression levels of housekeeping gene, GAPDH (data not shown). In the preparations of arteries treated with $0.3 \mu M$ doxorubicin for 7 days, the ratio of mRNA expressions encoding endothelial NO synthase (260 basepairs) to GAPDH (308 basepairs) showed no difference from that of the control arteries at 30 cycles of amplification ($0.25 \pm 0.01$ in control arteries and $0.23 \pm 0.01$ in the doxorubicin treated arteries, $n=5$, each). Additionally, in the preparations of BAECs treated with $0.3 \mu M$ doxorubicin for 12h, the ratio was no different from that of the control BAECs at 30 cycles of amplification ($0.71 \pm 0.01$ in control BAECs and $0.68 \pm 0.02$ in the doxorubicin treated BAECs, $n=6$, each).

Discussion

In the present study, we examined the toxicity of doxorubicin on vascular endothelium using the organ culture method, and revealed that doxorubicin induces DNA damages and
apoptotic cell death followed by the impairment of endothelium-dependent relaxation in a dose- and time-dependent manner.

In the arteries treated with 0.3 μM doxorubicin for 7 days, HE or TUNEL staining revealed that the nuclei of endothelial cells were damaged without any sign of damages to smooth muscle nuclei. Additionally, we compared the effects of doxorubicin in inducing apoptosis in BAECs and A7r5 smooth muscle cells and revealed that BAECs is more sensitive than A7r5 to the apoptotic effect of doxorubicin. This difference may be explained by the facts that the cell cycle in endothelial cells is more active than that in vascular smooth muscles [18, 19, 20], and that rapidly proliferating cells are more susceptible to doxorubicin and other chemotherapeutic agents.

In the arteries treated with 0.3 μM doxorubicin for 7 days, in which endothelial cells represented apoptotic changes, the endothelium-dependent relaxation caused by substance P remained intact. The relaxation in the cultured arteries is attributable mainly to NO production, because the relaxation was completely abolished by L-NMMA. To further elucidate the disassociation between the morphological and functional damages, we investigated the mRNA expression encoding endothelial NO synthase using semiquantitative RT-PCR. The results revealed that doxorubicin did not change the amounts of endothelial NO synthase mRNA. Similarly, the amount of endothelial NO synthase mRNA in BAECs treated with 0.3 μM doxorubicin for 12 h, in which DNA fragmentation was obvious, did not change from that of control BAECs. Biggiogera et al. (1998) indicated that the decrease in the amount of total RNA during apoptosis may be linked mainly to cellular extrusion of RNA rather than to deg-
radation by RNase [21], and suggested the possibility that RNA is intact during the early stage of apoptosis. These data support our findings that doxorubicin changed neither the endothelial NO synthase mRNA levels nor the endothelial function of relaxing vascular smooth muscle.

Treatment of arterial tissue with a higher concentration of doxorubicin (1 μM) for 3 days induced severe endothelial DNA-damages. These changes were similar to those of the arteries treated with 0.3 μM doxorubicin for 7 days. In spite of the DNA damages, endothelial function of relaxing smooth muscle remained normal as is the case with the artery treated with 0.3 μM doxorubicin for 7 days. In contrast, a longer (5 or 7 days) treatment with 1 μM doxorubicin denuded most of the endothelial cells followed by the impairment of endothelium-dependent relaxation. It has been reported that cells dying from apoptosis display DNA fragmentation at internucleosomal sites followed by altered nuclear morphology and finally loss of membrane integrity [22, 23]. Therefore, the doxorubicin-induced impairment of endothelium-dependent relaxation may result mainly from the induction of apoptosis followed by excoriation of endothelial cells. The present results regarding organ culture further demonstrated that apoptotic phenomena were observed not only in endothelial cells but also in smooth muscle cells. However, apoptosis in the smooth muscle cells was observed only at 1 μM doxorubicin-treatment for 5 or 7 days, suggesting that smooth muscle cells are more resistant to the apoptotic effect of doxorubicin.

Plasma levels of doxorubicin during chemotherapy have been estimated to be 0.2–1 μM [4]. Therefore, the concentrations of doxorubicin (0.3 and 1 μM) used for the present study are close to therapeutic concentrations, and the results obtained in this study may be clinically relevant to the vascular toxicity of doxorubicin.

In summary, this is the first report demonstrating the long-term effect of doxorubicin on vascular wall. We conclude that chronic treatment with doxorubicin at the therapeutic concentrations (0.3–1 μM) induces DNA damages and apoptotic morphological changes before the damaging vascular smooth muscle cells. The decrease in endothelium-dependent relaxation elicited by a higher concentration of doxorubicin and/or a longer period of treatment is mediated by excoriation of endothelial cells from the tunica interna secondary to the apoptotic reactions.

Dysfunctions of the circulatory system including hypotension and hypertension during the repetitive treatment with doxorubicin have been reported in vivo studies [8, 9, 10, 11]. However, it is considered that these circulatory dysfunctions are also caused by cardiac dysfunction by doxorubicin or compensative increase of cardiac output. Previously, we showed that chronic treatment with doxorubicin impairs vasoconstriction followed by induction of apoptosis in vascular smooth muscle [13], and an in vivo study indicated the similar effect of doxorubicin on vascular smooth muscle [24]. Taken together, these results suggest that long-term treatment with doxorubicin impairs the endothelial functions followed by the induction of apoptosis in endothelium, and then impairs the vasoconstriction of vascular smooth muscle. We could not reveal the direct interaction between the impairments of vasculature and imbalance of blood pressure, but these findings concerning doxorubicin toxicity to vascular wall should be useful in understanding the pathogenesis of dysfunction of circulatory system by doxorubicin during chemotherapy.
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