Expression of the *Nd1* gene is down-regulated by doxorubicin at post-transcriptional level

YASUYUKI TAKAMORI[1,2], YUJI MATSUDO[1], LISA FUJIMURA[3], MYENGMO KANG[1], YOSHITADA HARADA[2], HIDESHIGE MORIYA[2], TAKESHI TOKUHISA[1] and MASAHIKO HATANO[1,3]

[1]Departments of Developmental Genetics (H2), [2]Orthopedic Surgery (J1), Graduate School of Medicine; [3]Biomedical Research Center, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

Received April 11, 2006; Accepted June 14, 2006

Abstract. Doxorubicin is an anti-neoplastic agent with cardiotoxicity as a side effect. We previously demonstrated that doxorubicin treatment of mice resulted in a selective decrease in expression of the *Nd1* gene, which encoded a new kelch family actin binding protein in the heart. Here we show that doxorubicin treatment also reduced the Nd1 expression in various organs of mice and cultured cell lines. The treatment of Nd1-transgenic mice and Nd1-transfectants also selectively reduced levels of the exogenous Nd1 mRNAs, whose expression was under the control of various promoters. Furthermore, the doxorubicin-induced reduction of Nd1 mRNA expression in NIH3T3 cells was inhibited by treatment of these cells with cycloheximide. Thus, the doxorubicin treatment may specifically reduce the stability of Nd1 mRNA.

Introduction

Doxorubicin is an anthracycline antibiotic widely used for cancer chemotherapy. Its use is limited by the frequent incidence of dose-dependent chronic cardiomyopathy. Several mechanisms of the doxorubicin-induced cardiomyopathy are reported, including direct DNA damage and interference with DNA repair (1), the formation of free reactive oxygen radicals (2), and cytoskeletal changes (3). Cytoskeletal changes following doxorubicin treatment include reduction in the density of myofibrillar bundles, alterations on the Z-disc structure, and disarray and depolimerization of actin filaments (4). Several studies have suggested the interaction of doxorubicin with myofibrillar proteins in the etiology of doxorubicin cardiotoxicity (3,4). However, the molecular basis of doxorubicin-induced cardiotoxicity is not yet known.

We have recently identified a novel actin binding protein, Nd1, which belongs to a kelch family (5). The *Nd1* gene expresses two forms of splicing variants of the primary transcript. The long form of Nd1 (Nd1-L) contains a BTB/POZ domain in its N terminus and 6 kelch repeats in the C terminus. The short form (Nd1-S) has a BTB/POZ domain but lacks kelch repeats. *Nd1-L* mRNA is ubiquitously expressed in normal mouse tissues, most abundantly in the heart. Promoter analysis revealed that the expression of the *Nd1* gene was regulated as a housekeeping gene (6). The *Nd1-L* mRNA level did not change throughout the cell cycle nor after activation in lymphocytes. We used a mouse model of doxorubicin-induced cardiomyopathy to elucidate the function of Nd1 in the heart. Expression of Nd1-L in the heart decreased after administration of doxorubicin in mice. Furthermore, overexpression of Nd1-L in cardiac myocytes of transgenic mice reduced doxorubicin toxicity (Matsudo *et al*, unpublished data). Thus, Nd1-L plays an important role in protecting doxorubicin toxicity in cardiac myocytes (7). However, the mechanism responsible for these phenomena is not yet understood. In this study, we address a possible mechanism of alteration of *Nd1* gene expression by doxorubicin treatment. We show that the stability of *Nd1* mRNA may be specifically reduced in cultured cell lines and various mouse tissues by doxorubicin treatment.

Materials and methods

Northern blot analysis. Total RNAs were extracted from adult mouse tissues and some cultured cell lines with TRIzol reagent (GIBCO-BRL, Rockville, MD). Twenty micrograms of total RNA were loaded on a 1% agarose gel in MOPS buffer containing 6% formaldehyde, transferred to a nylon membrane (Roche Diagnostic, Mannheim, Germany), and fixed by cross linking with UV irradiation. The filter was hybridized with digoxigenin (DIG) labeled probe overnight at 50˚C. Following hybridization, the filter was washed twice with 0.1X SSC and 0.1% SDS at 58˚C for 15 min. The probe on the filter was detected with sheep anti-DIG Abs conjugated with alkaline phosphatase. The antibody detection reaction was performed using an enhanced chemiluminescent detection system (Roche Diagnostic). A 625 bp *Eco*RI-*Xba*I DNA fragment of *Nd1* cDNA including the BTB/POZ domain was

Correspondence to: Dr M. Hatano, Biomedical Research Center, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
E-mail: hatanom@faculty.chiba-u.jp

Abbreviations: CHX, cyclohexamide; HBx, hepatitis B virus X; IRP, iron regulatory protein; PBGD, porphobillinogen deaminase

Key words: Nd1, kelch family protein, doxorubicin, mRNA stability, post-transcriptional regulation

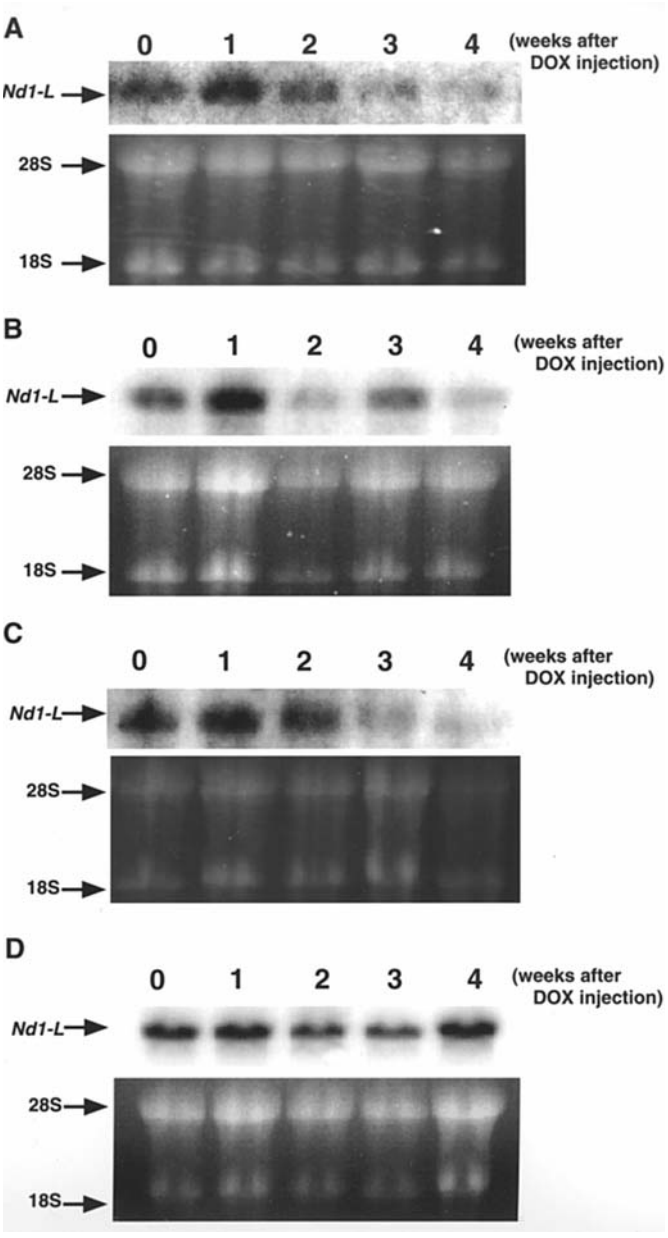

Figure 1. Stability of *Nd1-L* mRNA in various organs after doxorubicin treatment. Expression of *Nd1-L* mRNA in (A) skeletal muscle, (B) liver, (C) lung and (D) kidney after doxorubicin treatment. Six-week-old BDF1 mice were injected with doxorubicin, and the tissues were excised 0, 1, 2, 3 and 4 weeks after treatment. Northern blot analysis was performed using *Nd1* probe. Equal RNA loadings were documented by 18S and 28S ribosomal RNAs in the lower panel of each set.

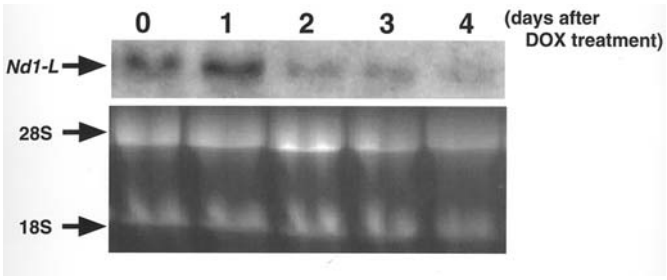

Figure 2. Expression of *Nd1-L* mRNA in NIH3T3 cells treated with doxorubicin. NIH3T3 cells were cultured in the presence of doxorubicin (0.1 μM). The cells were harvested 0, 1, 2, 3 and 4 days after treatment. Northern blot analysis was performed using *Nd1* probe. Equal RNA loadings were documented by 18S and 28S ribosomal RNAs in the lower panel.

used as an *Nd1* probe (5). The fragment was subcloned into pGEM-4Z (Promega) and labeled with the DIG labeling mixture by polymerase chain reaction (PCR) with T7 and SP6 primers. Cardiac α actin was obtained by means of reverse transcription (RT)-PCR from heart total RNA using specific oligonucleotide primer 5'-GGACAATTTCACGTT CAGCAGTGG-3' and 5'-CAAGGCGACGTAACACAGCT TTTC-3'. The PCR products were subcloned into the pGEM T-vector (Promega) and labeled with the DIG labeling mixture by PCR with T7 and SP6 primers.

Construction of HA epitope tagged Nd1-S expression plasmids. HA tagged Nd1-S expression plasmids (pCR-2HA-Nd1-S) were constructed by amplifying *Nd1-S* cDNA fragment containing open reading frame by PCR and ligating that to the *Eco*RI sites of pCR-2HA (8). Cadmium inducible expression plasmids (pSMT-2HA-Nd1-S) were described previously (5).

Cell culture and transfection. NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum at 37˚C under 5% CO_2. To establish stable transfectants, NIH3T3 cells were transfected with 10 μg of pSMT-2HA-Nd1-S along with 1 μg of pSV2Neo using electroporation and selected with 400 μg/ml G418 (Sigma). For induction of the SMT promoter, cells were cultured in the presence of 5 μM $CdCl_2$. WEHI231 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum at 37˚C under 5% CO_2. In some experiments, 0.1 μM of doxorubicin (Sigma) or 10 μg /ml of cycloheximide (CHX) (Sigma) were added to the culture.

Doxorubicin treatment of mice. A simple procedure for inciting doxorubicin-induced cardiomyopathy in mice has been previously described (9). Doxorubicin hydrochloride (Sigma) was dissolved in distilled water just before use. Mice were administered 5 mg/kg of doxorubicin intraperitoneally 4 times in 3- or 4-day intervals for 10 days. The care of all animals used in the present study was in accordance with Chiba University Animal Care guidelines.

Densitometry analysis. In some experiments, digitized measurements were investigated quantitatively by using NIH Image software.

Results

Reduction of Nd1-L mRNA expression in various mouse organs and cultured cell lines after doxorubicin treatment. The anthracycline antibiotic doxorubicin is an antineoplastic agent that has a cardiotoxic effect. We previously observed that the expression of *Nd1-L* mRNA in cardiac muscle decreased after doxorubicin treatment (Matsudo *et al*, unpublished data). In order to examine whether the effect of doxorubicin on *Nd1* mRNA expression was restricted to cardiac muscles, we examined the expression of *Nd1-L* in various organs of 6-week-old mice injected with doxorubicin 4 times. *Nd1-L* mRNA levels in the skeletal muscle, liver, lung (Fig. 1A-C)

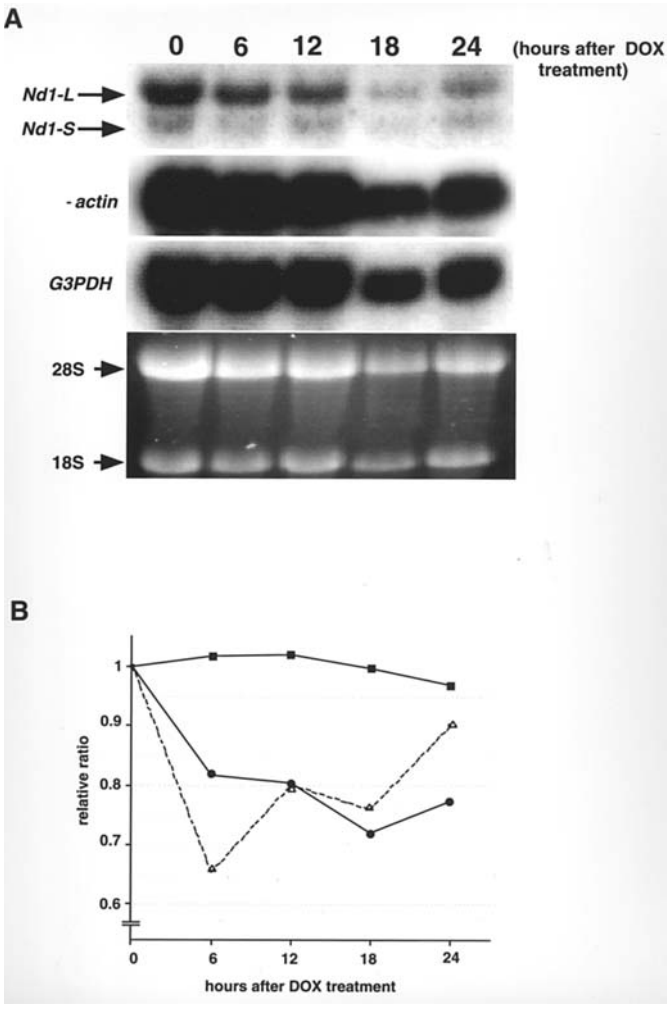

Figure 3. Expression of *Nd1-L* and *Nd1-S* mRNAs in WEHI231 cells treated with doxorubicin. (A) WEHI231 cells were cultured in the presence 0.1 μM of doxorubicin. The cells were harvested 0, 6, 12, 18 and 24 h after treatment. Northern blot analysis was performed using *Nd1*, *ß-actin*, and G3PDH as probes. Equal RNA loadings were documented by 18S and 28S ribosomal RNAs in the lower panel. (B) Digitized measurements were investigated quantitatively by using NIH Image software. *Nd1-L* (closed circles), *Nd1-S* (open triangles), and *ß-actin* (closed squares) gene expression was compared with *G3PDH*.

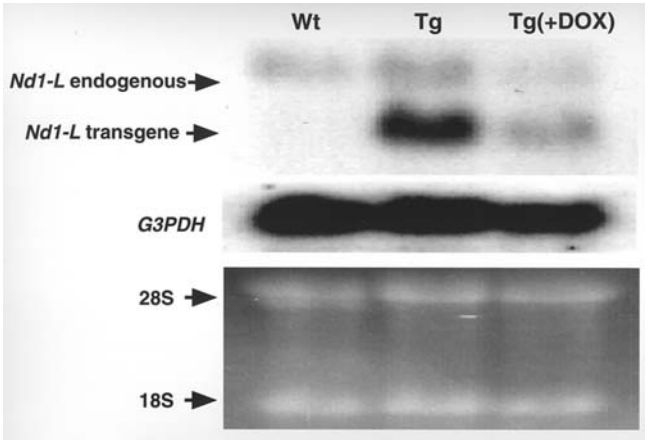

Figure 4. Alteration of cardiac gene expression after doxorubicin treatment. Nd1-L transgenic and wild-type mice were treated with a total dose of 20 mg/kg doxorubicin, and the hearts were excised after 4 weeks. Northern blot analysis was performed using *Nd1* and *G3PDH* probes. Equal RNA loadings were documented by 18S and 28S ribosomal RNAs in the lower panel. Wt, control littermate; Tg, Nd1-L Tg.

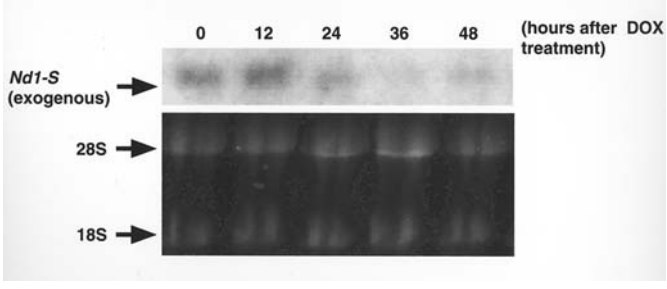

Figure 5. Stability of *Nd1-S* mRNA controlled by the sheep metallothionein promoter after doxorubicin treatment. NIH3T3 cells transfected with *Nd1-S* expression vector were cultured in the presence of doxorubicin (0.1 μM). The cells were harvested after 0, 12, 24, 36 and 48 h. Northern blot analysis was performed using *Nd1* probe. Equal RNA loadings were documented by 18S and 28S ribosomal RNAs in the lower panel.

and spleen (data not shown) transiently increased 1 week and then decreased 3 weeks after treatment. However, the expression did not change in the kidney during the course of treatment (Fig. 1D).

Next, we examined the effect of doxorubicin in cultured cell lines. NIH3T3 cells were cultured in the presence of 0.1 μM doxorubicin, and *Nd1-L* mRNA expression in these cells was examined. The expression transiently increased within 1 day and then decreased 2 days after treatment (Fig. 2). This reduction of mRNA expression was also observed in other cell lines such as WEHI231 (Fig. 3A) and 293T (data not shown). Thus, doxorubicin reduced expression of the *Nd1-L* gene in various organs and cell lines.

Since *Nd1* encodes two types of mRNA, *Nd1-L* and *Nd1-S* (6), we examined the effect of doxorubicin on *Nd1-S* expression. When WEHI231 cells were cultured in the presence of doxorubicin, the expression of both *Nd1-L* and *Nd1-S* mRNA was reduced within 24 h after treatment (Fig. 3A). Although actin mRNA decreased slightly, the reduction of *Nd1* mRNAs was much more distinct, suggesting that Nd1 mRNAs are susceptible to doxorubicin treatment (Fig. 3B).

Post-transcriptional regulation of Nd1 mRNA by doxorubicin treatment. We next examined whether the doxorubicin treatment affected transcription of the *Nd1* gene or *Nd1* mRNA stability. In order to examine the effect of doxorubicin on the *Nd1* promoter, we utilized Nd1-L transgenic mice (Nd1-L Tg), which carried the exogenous *Nd1-L* gene under the control of chicken ß actin promoter (Matsudo *et al*, unpublished data). The sizes of these endogenous and transgenic *Nd1-L* mRNAs were 3.2 kb and 3.1 kb, respectively, and these were distinguishable by Northern blot analysis. Amounts of both endogenous and exogenous *Nd1-L* mRNAs decreased 4 weeks after doxorubicin treatment (Fig. 4). To further examine the effect on the promoter of the *Nd1* gene, we used NIH3T3 cells stably transfected with the *Nd1-S* gene under the control of the sheep metallothionein promoter (10). When these transfectants were cultured in the presence of doxorubicin, the amount of exogenous *Nd1-S* mRNA decreased within 2

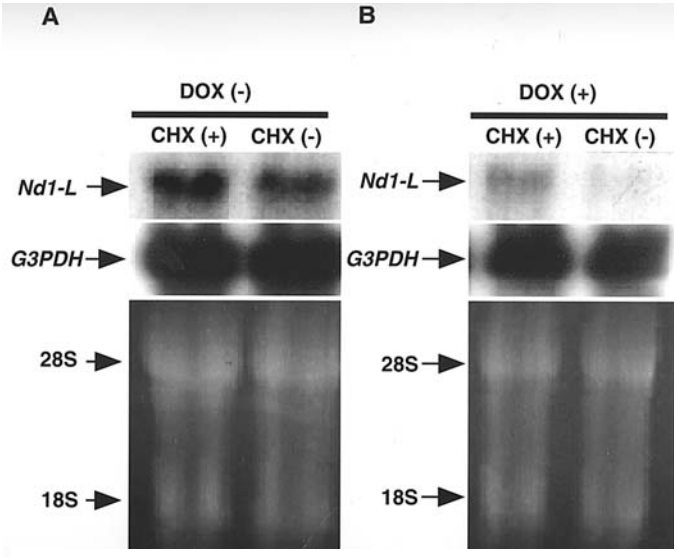

Figure 6. Effect of cycloheximide on the stability of *Nd1* mRNA. (A) NIH3T3 cells were cultured in the presence (CHX^+) or absence (CHX^-) of cycloheximide. The cells were harvested 4 h after treatment. (B) NIH3T3 cells were cultured in the presence of doxorubicin (0.1 μM) for 2 days with (CHX^+) or without (CHX^-) cycloheximide during the final 4 h. Northern blot analysis was performed using *Nd1* and *G3PDH* probes. Equal RNA loadings were documented by 18S and 28S ribosomal RNAs in the lower panel of each set.

days (Fig. 5). These data suggested that the reduction of *Nd1* mRNAs after doxorubicin treatment is not due to the negative effect of doxorubicin on the Nd1 promoter activity.

Finally, we examined the effect of CHX on the stability of *Nd1* mRNA. NIH3T3 cells were cultured with doxorubicin in the presence of CHX, which can stabilize any mRNAs sensitive to a short-lived RNase (11). The amount of *Nd1-L* mRNA in NIH3T3 cells cultured without doxorubicin did not change in the presence of CHX, indicating that *Nd1-L* mRNA itself was stable (Fig. 6A). When these cells were cultured with doxorubicin in the presence of CHX, the decrease of *Nd1-L* mRNA was inhibited while the amount of *G3PDH* was not affected (Fig. 6B). These data suggest that *Nd1-L* mRNA is actively degraded in the presence of doxorubicin. Thus, doxorubicin specifically affects the *Nd1* mRNA stability at post-transcriptional level in various organs and cell lines.

Discussion

In this study, we demonstrated the effect of doxorubicin on *Nd1* mRNA expression. Expression of *Nd1* mRNA decreased in cultured cell lines and in most mouse tissues after doxorubicin treatment. Since the decrease of *Nd1* mRNA was inhibited by the treatment of cells with CHX, doxorubicin affected the stability of *Nd1* mRNA, and the *Nd1* mRNA destabilization was dependent on *de novo* protein synthesis. We originally considered the possibility that the selectivity of doxorubicin on *Nd1* mRNA might in fact result from a nonspecific inhibition of gene expression and shorter half-lives for *Nd1* transcripts. However, *Nd1* mRNA was more sensitive to doxorubicin compared to other mRNAs from cytoskeletal and housekeeping genes.

Furthermore, the steady state of *Nd1* mRNA expression in NIH3T3 cells was not affected by the addition of CHX (Fig. 6A). Thus, there might be a specific mechanism to target the *Nd1* mRNA by doxorubicin.

Doxorubicin was reported to modify the expression of several genes. Alteration of the expression of these genes was responsible for doxorubicin-induced cardiomyopathy. Doxorubicin alters transcriptional events specific to the myocardium (12-15). Doxorubicin also inhibits the transcription of muscle specific genes in skeletal muscle cells without affecting the transcription of housekeeping genes (16). Doxorubicin induced an increased stability of porphobillinogen deaminase (PBGD) and GATA1 mRNAs in the post-transcriptionally K562 human erythroleukemic cell line (17). The transcription rate of PBGD and GATA1 was unchanged in the doxorubicin-treated cells. Expression of the hepatitis B virus X (HBx) gene mRNA transcript was upregulated after doxorubicin treatment in HBx expressing cells and HBx transgenic mice (18). The half-life of *HBx* mRNA was prolonged at post-transcriptional level in the presence of doxorubicin. The mechanisms of post-transcriptional modification by doxorubicin in these genes are not fully understood. Iron regulatory proteins (IRP) modulate the fate of mRNAs for transferrin receptor and ferritin (19-22). When the cell needs iron, IRP-1 binds to the iron responsive element in the mRNA for transferrin receptor, or increasing the stability of the mRNA. Doxorubicin irreversibly inactivates IRP in cardiomyocytes and affects the stability of transferrin receptor mRNA indirectly (23,24). It is possible that doxorubicin modifies expression of a protein that is responsible for stabilizing the *Nd1* mRNA specifically. Since both *Nd1-L* and *Nd1-S* mRNAs are decreased by doxorubicin, mRNA encoding the BTB/POZ domain may be responsible for stabilization of the mRNA. Further study is required to identify a factor to regulate *Nd1* mRNA stability induced by doxorubicin.

The anthracyclines produce a wide range of biochemical effects that have potentially toxic consequences for mammalian cells. There are several hypotheses to explain doxorubicin toxicity. One mechanism is mediated by the intercalation of the drug to DNA. This appears to be the major determinant of doxorubicin cytotoxicity to tumor cells. The other mechanism involves generation of oxygen free radicals. This appears to play a major role in the development of cardiomyopathy. We demonstrated that the cytotoxicity induced by doxorubicin is associated with alterations in the gene(s) important for the cytoskeletal structural integrity. Moreover, decrease of *Nd1* gene expression was observed after incubation of cultured cell lines with 0.1 μM doxorubicin, a concentration lower than the plasma peaks observed in patients after standard doses of the doxorubicin chemotherapy (7~10 μM) (25). It is possible that *Nd1* mRNA degradation is one of the primary targets of the doxorubicin cytotoxicity. The effects of doxorubicin on *Nd1* mRNA stability observed in this study facilitate the design of new anticancer chemotherapy. For example, selective inhibition of *Nd1* mRNA degradation in cardiomyocytes prevents doxorubicin-induced cardiomyopathy during cancer treatment. Alternatively, selective degradation of *Nd1-L* mRNA in tumor cells may provide an additive effect to cancer chemotherapy.

In summary, our experiments demonstrated that doxorubicin modifies the stability of *Nd1* mRNA post-

transcriptionally in various tissues. Our results present significant new information regarding the effects of doxorubicin on the regulation of *Nd1* mRNA, and this may be important in terms of designing novel antitumor therapies.

Acknowledgements

We thank Ms. H. Satake for her technical assistance, and Ms. N. Kakinuma for her secretarial services. This work was supported in part by a grant-in aid for scientific research from the Ministry of Education, Science, Technology, Sports and Culture of Japan.

References

1. Gewirtz DA: A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. Biochem Pharmacol 57: 727-741, 1999.
2. Olson RD and Mushlin PS: Doxorubicin cardiotoxicity: analysis of prevailing hypotheses. FASEB J 4: 3076-3086, 1990.
3. Colombo R, Milzani A, Necco A and Vailati G: Doxorubicin effects on contractile structures and molecules. Cytotechnology 3: 9-19, 1990.
4. Colombo R and Milzani A: How does doxorubicin interfere with actin polymerization? Biochim Biophys Acta 968: 9-16, 1988.
5. Sasagawa K, Matsudo Y, Kang M, Fujimura L, Iitsuka Y, Okada S, Ochiai T, Tokuhisa T and Hatano M: Identification of Nd1, a novel murine kelch family protein, involved in stabilization of actin filaments. J Biol Chem 277: 44140-44146, 2002.
6. Kang M, Matsudo Y, Sasagawa K, Tokuhisa T and Hatano M: Nd1, a novel murine Kelch family protein, may play the role of a housekeeping gene. Biochim Biophys Acta 1519: 167-174, 2001.
7. Fujimura L, Matsudo Y, Kang M, Takamori Y, Tokuhisa T and Hatano M: Protective role of Nd1 in doxorubicin-induced cardiotoxicity. Cardiovasc Res 64: 315-321, 2004.
8. Akiba H, Nakano H, Nishinaka S, Shindo M, Kobata T, Atsuta M, Morimoto M, Ware CF, Malinin NL, Wallach D, Yagita H and Okumura K: CD27, a member of the tumor necrosis factor receptor superfamily, activates NF-kappaB and stress-activated protein kinase/c-Jun N-terminal kinase via TRAF2, TRAF5, and NF-kappaB-inducing kinase. J Biol Chem 273: 13353-13358, 1998.
9. Kim C, Nam SW, Choi DY, Park ES, Jhoo WK and Kim HC: A new antithrombotic agent, aspalatone, attenuated cardiotoxicity induced by doxorubicin in the mouse; possible involvement of antioxidant mechanism. Life Sci 60: 75-82, 1997.
10. Peterson MG and Mercer JF: Structure and regulation of the sheep metallothionein-Ia gene. Eur J Biochem 160: 579-585, 1986.
11. Brewer G and Ross J: Regulation of c-myc mRNA stability *in vitro* by a labile destabilizer with an essential nucleic acid component. Mol Cell Biol 9: 1996-2006, 1989.
12. Jeyaseelan R, Poizat C, Wu H-Y and Kedes L: Molecular mechanisms of doxorubicin-induced cardiomyopathy: selective suppression of REISKE iron-sulfer protein, ADP/ATP translocase, and phosphofructokinase genes is associated with ATP depletion in rat cardiomyocytes. J Biol Chem 272: 5828-5832, 1997.
13. Kurabayashi M, Jeyaseelan R and Kedes L: Antineoplastic agent doxorubicin inhibits myogenic differentiation of C2 myoblasts. J Biol Chem 268: 5524-5529, 1993.
14. Kurabayashi M, Jeyaseelan R and Kedes L: Doxorubicin represses the function of the myogenic helix-loop-helix transcription factor MyoD. Involvement of Id gene induction. J Biol Chem 269: 6031-6039, 1994.
15. Kurabayashi M, Dutta S, Jeyaseelan R and Kedes L: Doxorubicin-induced Id2A gene transcription is targeted at an activating transcription factor/cyclic AMP response element motif through novel mechanisms involving protein kinases distinct from protein kinase C. Mol Cell Biol 15: 6386-6397, 1995.
16. Ito H, Miller SC, Billingham ME, Akimoto H, Torti SV, Wade R, Gahlmann R, Lyons G, Kedes L and Torti FM: Doxorubicin selectivity inhibits muscle gene expression in cardiac muscle cells *in vivo* and *in vitro*. Proc Natl Acad Sci USA 87: 4275-4279, 1990.
17. Morceau F, Chenais B, Gillet R, Jardillier JC, Jeannesson P and Trentesaux C: Transcriptional and posttranscriptional regulation of erythroid gene expression in anthracycline-induced differentiation of human erythroleukemic cells. Cell Growth Differ 7: 1023-1029, 1996.
18. Yun C, Lee JH, Wang JH, Seong JK, Oh SH, Yu DY and Cho H: Expression of hepatitis B virus X (HBx) gene is up-regulated by adriamycin at the post-transcriptional level. Biochem Biophys Res Commun 296: 1157-1163, 2002.
19. Klausner RD, Rouault TA and Harford JB: Regulating the fate of mRNA: the control of cellular iron metabolism. Cell 72: 19-28, 1993.
20. Beinert H and Kennedy MC: Aconitase, a two-faced protein: enzyme and iron regulatory factor. FASEB J: 71442-71449, 1993.
21 Hentze MW and Kuhn LC: Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. Proc Natl Acad Sci USA 93: 8175-8182, 1996.
22. Cairo G and Pietrangelo A: Iron regulatory proteins in pathobiology. Biochem J 352: 241-250, 2000.
23. Minotti G, Ronchi R, Salvatorelli E, Menna P and Cairo G: Doxorubicin irreversibly inactivates iron regulatory proteins 1 and 2 in cardiomyocytes: evidence for distinct metabolic pathways and implications for iron-mediated cardiotoxicity of antitumor therapy. Cancer Res 61: 8422-8428, 2001.
24. Kwok JC and Richardson DR: Unexpected anthracycline-mediated alterations in iron-regulatory protein-RNA-binding activity: the iron and copper complexes of anthracyclines decrease RNA-binding activity. Mol Pharmacol 62: 888-900, 2002.
25. Gianni L, Vigano L, Locatelli A, Capri G, Giani A, Tarenzi E and Bonadonna G: Human pharmacokinetic characterization and *in vitro* study of the interaction between doxorubicin and paclitaxel in patients with breast cancer. J Clin Oncol 15: 1906-1915, 1997.