Protective role of Nd1 in doxorubicin-induced cardiotoxicity

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Abstract

Objective: The Nd1 gene, encodes a novel kelch family protein, is expressed ubiquitously in mouse tissues. In vitro studies suggest that Nd1 protein, which binds to actin filaments, functions as a cytoskeletal stabilizer. In order to elucidate a physiological function of Nd1 in vivo, we generated Nd1-deficient (Nd1-/-) mice.

Methods: We developed Nd1-/- mice by standard gene targeting technique. Cardiac function was studied in wild type and Nd1-/- mice.

Results: Nd1-/- mice were viable and no gross anatomical abnormality was observed after birth. When mouse embryonic fibroblasts were cultured in the presence of cytochalasin D or doxorubicin, the number of apoptotic cells in the Nd1-/- cell culture was larger than that in the wild-type cell culture. Furthermore, Nd1-/- mice were sensitive to doxorubicin-induced cardiotoxicity with increased numbers of cardiomyocytes apoptosis.

Conclusions: Although Nd1 is dispensable for normal mice development, Nd1 plays a protective role in doxorubicin-induced cardiotoxic responses.

Keywords: Actin-binding protein; Doxorubicin; Kelch family protein; Nd1-deficient mice

1. Introduction

The kelch family protein is one of the actin-binding proteins defined by an approximately 50-amino-acid motif repeat [1,2]. The kelch motif was originally discovered as a six-fold tandem element in the sequence of the Drosophila kelch ORFI protein. The kelch repeat motif appears to function as a novel actin-binding domain [3]. More than 30 proteins containing kelch repeats have been identified in a diverse set of organisms, including viruses, yeast, C. elegans, Drosophila, and mammals [4]. The kelch repeat motifs predict a conserved tertiary structure, a β-propeller. This module may have functional significance in binding actin, protein folding, or protein–protein interactions. In addition to the kelch repeat motif, most kelch family proteins also possess a 120-amino-acid motif referred to as the BTB/POZ domain at the extreme amino terminus of the protein [5,6]. The BTB/POZ domain is found at the amino terminus of several transcription factors in Drosophila, viruses, and mammals [7–12]. It has been reported that BTB/POZ domains mediate homo- and heterodimerization in vitro and the formation of multimeric complexes in vivo [5,6].

We have identified a novel kelch family gene that encodes two forms of proteins, Nd1-L and Nd1-S [13,14]. Nd1-L mRNA is expressed ubiquitously in normal mouse tissues, most abundantly in the heart. Nd1-L binds to actin filaments through its kelch repeats. Overexpression of Nd1-L in NIH3T3 cells delayed cell growth by affecting the transition of cytokinesis. Furthermore, the overexpression prevented the cells from cell death induced by actin destabilizing agents. Thus, Nd1-L functions as a stabilizer of actin filaments and as an actin-binding protein. We also examined a role of Nd1-L...
in the heart in vitro and in vivo. We found that overexpression of Nd1-L in cardiomyocytes protected these cells from doxorubicin-induced cardiotoxicity (Matsumoto et al., submitted for publication).

In *Drosophila*, the kelch protein is a component of ring canal and cross-links actin bundles. Loss of the kelch-containing protein, tea1, in yeast exhibits a defect in cell division [16]. While growing numbers of kelch family member proteins have been identified in mammals, the physiological roles of kelch family proteins are not fully understood. In order to elucidate a physiological function of Nd1 proteins, we created Nd1-deficient (Nd1−/−) mice. Nd1−/− mice were viable, and no gross anatomical abnormality was observed. However, Nd1−/− mice were sensitive to doxorubicin-induced cardiotoxicity. The physiological role of Nd1 in the cardiomyocytes is discussed.

2. Methods

2.1. Gene targeting in embryonic stem cells

A 9.0-kb fragment from the *Xho1* site in 5′ flanking region of the *Nd1* gene to the *Not1* site in exon 9 was replaced by a neomycin resistant gene cassette (pMC1-neo). For negative selection, a herpes simplex thymidine kinase gene cassette was fused at the 3′ end of the long arm. R1 embryonic stem cells were transfected with the linealized targeting vector by electroporation and subjected to positive and negative selection using G418 and gancyclovir for 14 days. Approximately 90 clones were examined by Southern blots and the homologous recombination was detected in two clones. Two independent targeted clones were used to generate chimeric mice using the aggregation method [17] with some modification. Tail DNAs from agouti pups obtained from mating with C57BL/6 mice were analysed by Southern blots. Homozygous mutant pups were generated by intercrossing heterozygous mutant mice. Specific pathogen-free C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). All experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committee at Graduate School of Medicine, Chiba University.

2.2. Southern blots analysis

Southern blot analysis was performed as described previously [17]. Briefly, genomic DNA isolated from the mutant offspring was digested with *Pst1*, separated on a 1% agarose gel, and transferred to a nylon membrane (Amersham Pharmacia) and fixed by cross-linking with UV irradiation and by baking at 80 °C for 5 min. The filter was pre-hybridized for 2 h and hybridized overnight at 42 °C in 50% formamide hybridization buffer with the probe indicated as probe in (A). The position of the hybridizing fragments for the wild type (2.5 kb) and the targeted allele (2 kb) is shown. (C) Northern blot analysis of *Nd1* mRNA digested with *Pst1*. A probe used for Southern screening is indicated as probe in (A). The position of the hybridizing fragments for the wild type (2.5 kb) and the targeted allele (2 kb) is shown. (C) Northern blot analysis of *Nd1* mRNA from the heart from 4-week-old mice. The probe revealed the authentic mRNA (3.2 kb) prominent in Nd1+/+, diminished in Nd1+/−, and undetectable in Nd1−/− mice. The lower part of the panel shows the loading control detected with the G3PDH probe.

Fig. 1. Targeted disruption of the *Nd1* gene. (A) The *Nd1* locus containing sixteen exons (closed boxes) and a portion of 5′ and 3′ flanking regions is shown (top). The targeting vector (middle) was designed to replace a DNA segment through exon 1 to exon 9 by a neomycin resistant gene cassette (NEO). A herpes simplex thymidine kinase gene cassette was fused at the 3′ end of the long arm. R1 embryonic stem cells were transfected with the linealized targeting vector by electroporation and subjected to positive and negative selection using G418 and gancyclovir for 14 days. Approximately 90 clones were examined by Southern blots and the homologous recombination was detected in two clones. Two independent targeted clones were used to generate chimeric mice using the aggregation method [17] with some modification. Tail DNAs from agouti pups obtained from mating with C57BL/6 mice were analysed by Southern blots. Homozygous mutant pups were generated by intercrossing heterozygous mutant mice. Specific pathogen-free C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). All experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committee at Graduate School of Medicine, Chiba University.
0.5% SDS, 1% blocking reagent and 15 ng/ml of probe. Following hybridization, the filter was washed twice for 5 min with 2×SSC and 0.1% SDS at room temperature and twice for 15 min with 0.1×SSC and 0.1% SDS at 55 °C. The probe was detected with sheep anti-digoxigenin (DIG) antibody conjugated with alkaline phosphatase (Roche Diagnostics). The antibody reaction was performed using an enhanced chemiluminescent detection system (Roche Diagnostics). As a probe, a 0.5-kb DNA fragment, which is external to the targeting vector, was labeled with DIG (Roche Diagnostics) by polymerase chain reaction (Fig. 1A). The probe detected the wild type allele as a 2.5-kb fragment and the mutant allele as a 2.0-kb fragment.

2.3. Northern blot analysis

Total RNAs were extracted from mouse tissues or mouse embryonic fibroblasts (MEFs) with the Trizol reagent (Life Technologies, Gaithersburg, MD). Northern blot analysis was done as described previously [18]. Briefly, total RNAs (20 μg) were electrophoresed through a 1.0% agarose gel containing formaldehyde, transferred to a nylon membrane. The filter was hybridized with a DIG-labeled probe and followed by the method described for Southern blot analysis. A 625-bp fragment (+30 to +655) of the Nd1 cDNA, including a BTB/POZ domain, was used as a POZ probe [14]. This fragment was subcloned into pGEM-4Z and labeled by DIG using PCR with T7 and SP6 primers. Bax and bcl-xL probes were described previously [19].

2.4. Doxorubicin treatment of mice

A simple procedure for inducing doxorubicin-induced cardiomyopathy in mice has been previously described [20]. Doxorubicin hydrochloride (Sigma) was solved in DDW just before use. Ten weeks old mice were administered 5 mg/kg of doxorubicin intraperitoneally four times in 3 or 4 days interval for 2 weeks.

2.5. Echocardiography

Mice were anesthetized with pentobarbital sodium (0.08 mg/g bw) (Abbott, Japan) intraperitoneally. M-mode echocardiography was performed using a commercially available system (SONOS5500, Phillips Medical system).

2.6. TdT-mediated dUTP-biotin Nick Labeling (TUNEL) Assay

Animals (6-month-old) were perfused with a solution of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Organs were dissected and postfixed with 4% paraformaldehyde for overnight. The tissues were equilibrated with 20% sucrose, sectioned at 5 μm on a cryostat. TUNEL assay was carried as described with slight modification [21]. The tailing reaction was carried out in TdT buffer in the presence of dUTP-biotin for 1 h at 37 °C. Signals were visualized by avidin-FITC (The Mebstein Apoptosis kit, Medical and Biological Laboratories, Nagoya, Japan). Sections were counter-stained with Propidium Iodide.

2.7. Histopathological preparations

Organs were dissected and postfixed with 4% paraformaldehyde for overnight. The tissues were processed through paraffin embedding using standard procedures. A transverse section of heart was made at about 1/3 of total length from the apex. Sections (5 μm) were stained with hematoxylin and eosin.

2.8. Cell culture

Mouse embryonic fibroblasts (MEFs) were established by harvesting embryos at embryonic day 13 from Nd1+/− crosses. Head and liver were removed from embryos.
and the remaining embryonic tissues were tripiniized at 37 °C for 30 min. The disrupted tissues were plated in DMEM supplemented with 15% FCS (Sigma) and cultured at 37 °C in 5% CO₂. In some experiments, cytochalasin D (Sigma) or doxorubicin (Sigma) was added to the culture.

2.9. Annexin V assay

MEFs were treated with cytochalasin D (10 μM) or doxorubicin (2 μM). Cells were collected 8 and 24 h after treatment, stained with annexin V coupled to fluorescein isothiocyanate (Becton Dickinson) and propidium iodide in accordance with the manufacturer’s instructions, and analysed on a FACScan (Becton Dickinson) with the use of CellQuest software. The percentage of apoptotic cells was calculated by scoring for cells that were annexin V positive.

2.10. Statistical analysis

Data presented represents the mean±S.D. of three independent experiments. Statistical analysis was performed by unpaired t test. P values of <0.05 were considered to be significant.

3. Results

3.1. Generation of Nd1−/− mice

Exons from 1 to 9 including the ATG translation start codon and the 5' flanking region of the Nd1 gene was replaced by the neomycin resistant gene in embryonic stem cells (Fig. 1A). Heterozygous mutant (Nd1+/−) mice showed no abnormality up to 2 years of life. Nd1+/− mice from the F1 generation were interbred to obtain Nd1−/− mice and their progeny were genotyped by Southern blot analysis (Fig. 1B). Neither Nd1-L nor Nd1-S mRNA was detected in total RNA from any tissues of Nd1−/− mice examined by Northern blot analysis (Fig. 1C and data not shown). Analysis of over 200 offsprings revealed that the number of Nd1−/− mice was approximately 10% lower than the expected Mendelian inheritance (−/−; 34/213: 16.0%, +/−; 120/213: 56.3%, +/-; 59/213: 27.7%). Growth curves and body weight of surviving Nd1−/− mice were indistinguishable from Nd1+/− and Nd1+/+ littermates (data not shown), and Nd1−/− mice were fertile. Although Nd1 is expressed in various organs ubiquitously, histological analysis of these tissues from Nd1−/− mice revealed no abnormality (data not shown).

![Fig. 3. Effect of doxorubicin on survival of Nd1−/− mice.](https://academic.oup.com/cardiovascres/article-abstract/64/2/315/321931)

(A) Survival rates for mice injected with doxorubicin. Ten-week-old Nd1−/− (n=8) and control littermates (n=11) were injected with 5 mg/kg of doxorubicin (DOX) four times in 3-day interval intraperitoneally and bred under normal condition. Kaplan-Meier survival curves represent significantly better survival (p<0.01) in control littermates. (B) Hematoxylin–eosin staining of the heart of Nd1−/− mouse treated with (DOX+) or without (DOX−) doxorubicin (2 weeks after injection). Arrows indicate vacuolization of cardiomyocytes.
3.2. Nd1−/− mouse embryonic fibroblasts (MEFs) are susceptible to stress induced by actin destabilization

Since Nd1-L binds to actin cytoskeleton, we examined whether lack of the Nd1 gene affected some functions related to actin organization using MEFs. Growth of Nd1−/− MEFs was comparable to that from wild type MEFs. Both Nd1−/− and wild type MEFs could be cultured more than 10 passages and no difference was observed between them. When cultured in the presence of cytochalasin D, apoptotic cells significantly increased in Nd1−/− MEFs but not in the control MEFs 24 h after treatment (Fig. 2A). Doxorubicin is an anti-neoplastic agent known to affect actin cytoskeleton [22]. Treatment of Nd1−/− MEFs with doxorubicin also induced a significant increase of apoptotic cells 8 and 24 h after treatment (Fig. 2B). Thus, Nd1−/− fibroblast cells were susceptible to stress induced by actin destabilizing agents.

In order to elucidate a molecular mechanism of doxorubicin-induced apoptosis in Nd1−/− MEFs, we examined expression of apoptosis-related genes in MEFs before and after doxorubicin treatment (Fig. 2C). Dose-dependent increase in both bax and bcl-xL mRNAs was observed up to 0.5 μM of doxorubicin. Interestingly, expression of bax and bcl-xL mRNAs was up-regulated in Nd1−/− MEFs even before doxorubicin treatment. Furthermore, bax expression in Nd1−/− MEFs was constantly higher than that of wild type MEFs. In contrast, there were no differences in expression of bcl-2 and p53 genes between wild type and Nd1−/− MEFs at any time point examined (data not shown).

3.3. Susceptibility of Nd1−/− mice to doxorubicin-induced cardiotoxicity

Since Nd1 was most abundantly expressed in the heart of normal mice, we examined the cardiac function of Nd1−/− mice. Nd1−/− mice developed normally and there are no gross abnormalities up to 12 months of age. Histological analysis of hearts from 3- to 6-month-old Nd1−/− mice did not show cardiac hypertrophy, fibrosis or infiltration of inflammatory cells. Cardiac function of 7-week-old Nd1−/− and control littermates was examined by echocardiography. There were no significant differences in IVSTd (WT: 1.07 vs. Nd1−/−: 1.02), LVPWTd (WT: 1.02 vs. Nd1−/−: 1.08), and LVDd/Ds (WT: 3.2/1.85 vs. Nd1−/−: 3.05/1.84) between Nd1−/− and control mice. Ten-week-old Nd1−/− and control mice were injected with 5 mg/kg of doxorubicin four times in 3 days interval intraperitoneally. More than 60% of the control mice survived 4 weeks after initial doxorubicin injection (Fig. 3A). In contrast, more than 90% of the Nd1−/− mice died within 4 weeks after doxorubicin treatment. Most of the doxorubicin-treated mice found dead and they were found to have massive bilateral pleural effusion and ascites, suggest-

![Fig. 4. M-mode echocardiography analysis. (A) Movement of left ventricular anterior and posterior wall was assessed in mice 2 weeks after doxorubicin treatment (5 mg/kg). Upper figure indicates wild type mouse and lower figure indicates Nd1 deficient one. The results are representative of five independent experiments. (LVDd: Left Ventricular Diameter diastolic, LVDs: Left Ventricular Diameter systolic). (B) Cardiac function of 4- to 5-month-old Nd1−/− and control mice was examined by echocardiography. Left ventricular ejection fraction (LVEF) was assessed before and after 2 weeks doxorubicin treatment. Results represent the mean± S.D. in three to five animals in each group (p<0.05).](https://academic.oup.com/cardiovascres/article-abstract/64/2/315/321931)
ing that mice had congestive heart failure. Morphological analysis of the hearts revealed that vacuolization and edema of cardiomyocytes were present in the heart of DOX treated Nd1−/− mice (Fig. 3B). We next examined cardiac function of the mice 2 weeks after initial doxorubicin treatment by echocardiography (Fig. 4). In Nd1−/− mice, movement of left anterior ventricular wall was poor and left ventricular ejection fraction (LVEF) was decreased, which often found in doxorubicin-induced cardiotoxicity [23]. On the other hand, cardiac function of wild type mice was not affected at this stage.

3.4. The number of apoptotic cardiomyocytes increased in Nd1−/− mice treated with doxorubicin

A recent finding indicates the importance of cardiomyocytes apoptosis in doxorubicin-induced cardiotoxicity [24]. In order to examine an effect of doxorubicin on cardiomyocytes apoptosis in Nd1−/− mice, TUNEL analysis was performed 24 h after a single doxorubicin injection (20 mg/kg). Compared to the control littersmates, significant increase in TUNEL-positive apoptotic cardiomyocytes was detected in Nd1−/− mice (Fig. 5).

4. Discussion

We have created Nd1−/− mice by the gene targeting method. Although Nd1 was expressed ubiquitously [14], Nd1−/− mice are fertile and have no apparent anatomical abnormalities. Thus, Nd1 is not essential for normal development and morphogenesis. The number of Nd1−/− mice delivered from Nd1+/− matings was lower than expected one at 4 weeks of age. Nd1−/− MEFs proliferate normally and could be cultured over 10 passages. Since Nd1−/− MEFs were more sensitive to cytochalasin D and doxorubicin than control MEFs, Nd1 protein may play a protective role against cytoskeletal stress. Rapid cell proliferation and morphological changes take place during embryogenesis. Although we could not see any abnormalities in neonatal period and no sudden death was observed after delivery in Nd1−/− litters, some Nd1−/− mice may not survive under these physiological stresses.

Since Nd1 is expressed most abundantly in the heart, we examined a role of Nd1 in cardiomyocytes using an animal model of cardiomyopathy induced by doxorubicin. The cardiomyopathy is known to associate with alterations in expression of genes important for the structural integrity [25–27]. Nd1 mRNA expression in the heart was also reduced after doxorubicin administration in mice (Matsuo et al., submitted for publication). Since Nd1−/− mice are susceptible to doxorubicin-induced cardiotoxicity, Nd1 together with other cellular cytoskeletal proteins may play a protective role against the cardiotoxicity. It is becoming clear that apoptosis of cardiomyocytes plays a critical role in the onset of cardiomyopathy [24,26]. Several studies suggest that the cardiomyocytes apoptosis induced by doxorubicin was attributed to H2O2 formation [28–30]. H2O2 exposure promotes translocation of the proapoptotic proteins such as Bax and Bad to the mitochondria, formation of heterodimers with Bcl-xL or Bcl-2, and subsequent release of cytochrome c in cardiomyocytes. Interestingly, expression of bax and bcl-xL mRNA increased in Nd1−/− cardiomyocytes. Link between cytoskeletal protein and apoptosis regulators such as Bim was reported [31]. Although molecular link between Nd1 and apoptosis regulators is not clear at this moment, Nd1 protein may interfere with apoptotic signals induced by doxorubicin treatment. Alternatively, Nd1 may directly protect cells against oxidative stresses like Keap1, other mammalian kelch family protein [32].

Recent studies suggest the critical role of gp130 and STAT3 signaling pathway for the protection of cardiomyocyte apoptosis and cardiomyopathy induced by doxorubicin treatment [20,33]. Expression of gp130 and STAT3 was examined in Nd1−/− cardiomyocytes after doxorubicin treatment, but no significant difference was observed between the Nd1−/− mice and control littersmates (data not shown). Thus, the protective mechanism of cardiomyocytes from doxorubicin by Nd1 is independent of the gp130 signaling pathway. Additional work is required to elucidate a signaling pathway related to Nd1 and actin cytoskeleton.

This is the first report that describes mammalian kelch family protein-deficient mice. In Kelch protein-deficient Drosophila, oogenesis is perturbed [3]. Drosophila kelch protein is essential for formation of the structure of cytoplasmic bridge called ring canal [34]. Various kelch-repeat proteins participate in the coordination of morphology and growth [35]. In S. pombe, tea1 [35], a kelch containing protein, deficiency causes cell division abnormalities. In S. cerevisiae, disruption of the KEL1 gene results in impaired cell fusion and alteration of cell morphology [36]. In these organisms, kelch family proteins play a critical role in basic cellular events. Although Nd1 expressed ubiquitously, Nd1−/− mice develop normally up to 1 year. This suggests the redundancy of kelch family proteins in mammals. Alternatively, Nd1 protein is not essential for normal development but may be required when cells are under certain stress that causes actin disorganization. Gene disruption study of other mammalian kelch family is required to determine the physiological function of mammalian kelch family.

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References


