Abstract

Background: This study was conducted to test the effects of thioredoxin (Trx)1 down-regulation in Trx2 transgenic [Tg(TXN2)^+/0] mice on lifespan and age-related diseases. Our previous study with Tg(TXN2)^+/0 mice showed that mitochondrial Trx overexpression produced minimal life-extending effects with a slightly elevated severity of lymphoma and another study with a limited number of Trx1KO mice showed that there was a slight reduction of neoplastic lesions. Thus, this study was aimed to test if reduced Trx1 expression in combination with elevated Trx2 has beneficial effects on lifespan in mice by attenuating age-related diseases, specifically cancer.

Methods: Trx2 hemizygous transgenic and Trx1 heterozygous knockout mice [Tg(TXN2)^+/0 x Trx1KO] were generated for survival and cross-sectional pathology experiments.

Results: Tg(TXN2)^+/0 x Trx1KO mice showed significantly higher (approximately 1.5- to 3-fold) Trx2 levels and significantly less (approximately 50% less) Trx1 levels in all of the tissues we examined compared to wild-type (WT) littermates. Trx1 down-regulation along with Trx2 overexpression did not change the levels of glutathione or other major antioxidant enzymes. Male Tg(TXN2)^+/0 x Trx1KO mice demonstrated only a slight extension of lifespan in the early part of life and no significant effects on the later part of life were observed, which was similar to our previous study with Tg(TXN2)^+/0 mice. Tg(TXN2)^+/0 x Trx1KO mice had similar tumor burden, disease burden, incidence and severity of lymphoma, and severity of glomerulonephritis compared to WT mice at 22-26 months.

Conclusions: Our findings suggest that the combined Trx down-regulation in cytosol along with upregulation in mitochondria of Tg(TXN2)^+/0 x Trx1KO mice did not provide beneficial effects on aging, i.e., extend the lifespan or reduce age-related pathology compared to WT mice.

Keywords: Thioredoxin, transgenic mouse, knockout mouse, aging, cancer

Introduction

Possible beneficial roles of thioredoxin (Trx) in aging and age-related diseases have been investigated in multiple studies, including several performed in our laboratory [1-5]. Trx is of interest in aging research because of its unique ability to alter oxidative stress and redox state, thereby affecting redox-sensitive signaling and subsequent effects on pathophysiology. This small protein (12 kDa) has two redox-active cysteine residues in the active center (Cys-Gly-Pro-Cys). It plays an important role as the reductant for various molecules via thiol-disulfide exchange reactions [6-12]. These rapid and readily reversible reactions are ideal to control protein function by changing the redox state of structural or catalytic SH groups of proteins.
Our laboratory has conducted aging studies with mice overexpressing or downregulating Trx in the cytosol and/or mitochondria to test the effects of Trx on aging and age-related diseases [2-5]. First, we examined the effects of Trx1 (cytosolic Trx) overexpression on aging with two lines of transgenic mice [Tg(act-TXN)−/0 and Tg(TXN)−/0 mice] [2, 3]. Survival experiments with these mice showed that overexpression of Trx1 slightly extended the earlier part of life but did not show significant effects on maximum lifespans. Interestingly, Trx1 overexpression was accompanied by accelerated cancer development in the later part of life [2, 3].

These initial studies with Trx1 transgenic mice led us to conduct another survival study with mice overexpressing Trx in mitochondria (Trx2) because the importance of antioxidant overexpression in mitochondria in aging was strongly suggested by the study by Schriner et al [13]. The survival study using Trx2 transgenic mice [Tg(TXN2)−/0] showed that Trx overexpression in mitochondria had a slight but not significant extension (approximately 8-9%) of mean, median, and 10th percentile lifespans compared to WT mice. Tg(TXN2)−/0 mice also showed a slightly higher severity of lymphoma compared to WT mice [4]. Because we have not observed significant effects on lifespan, specifically in the latter part of life, by Trx overexpression in cytosol or mitochondria alone, we further tested if it is necessary to have changes in the levels of Trx in both the cytosol and mitochondria. One of the potential reasons Trx2 overexpression did not significantly extend lifespan could be due to its effects on age-related tumor formation [4]. Both Trx1 and Trx2 transgenic mice showed a tendency to accelerate tumor development with age [2-4]. Our previous study with Trx1KO mice showed that reduced Trx1 did not change the lifespan, i.e., no extension or shortening; however, there was a subtle reduction of age-related cancer based on limited cross-sectional pathological analyses [5]. These observations led us to the hypothesis that reducing Trx1 could prevent age-related tumor development in Tg(TXN2)−/0 mice, resulting in an extended lifespan compared to WT mice.

Thus, the purpose of this study is to examine the effects of reduced Trx1 along with Trx2 overexpression on aging and age-related diseases. We conducted a survival study using mice overexpressing Trx2 and down-regulating Trx1 [Tg(TXN2)−/0 x Trx1KO mice]. We report that the combined Trx down-regulation in cytosol and upregulation in mitochondria did not extend the lifespan, although these mice did show a slight extension (approximately 30.7%) of lifespan in the early stage of life. Age-related pathology in Tg(TXN2)−/0 x Trx1KO mice was similar to WT mice. Therefore, our results suggest that reduced Trx1 along with Trx2 overexpression did not have significant effects on lifespan or age-related diseases including tumor development in male C57BL/6 mice.

Materials and Methods

Animals and animal husbandry
Tg(TXN2)−/0 mice were generated using the human thioredoxin 2 gene [a PAC clone (RP5-1119A7), Children’s Hospital Oakland Research Institute’s (CHORI) BACPAC Resources Center (BPRC), Oakland, CA] as previously described [4]. Trx1 heterozygous knockout mice were generated with an ES clone purchased from Lexicon Pharmaceuticals, Inc. (OST452454) as previously described [5]. Male heterozygous Trx1KO mice were crossed to female hemizygous Trx2 transgenic mice [Tg(TXN2)−/0] to generate Tg(TXN2)−/0 x Trx1KO and WT control mice. These mice were fed ad libitum with a commercial chow (Teklad Diet LM485; Madison, WI) and free access to acidified (pH = 2.6-2.7) filtered reverse osmosis water. The amount of chow removed from the cage hopper and the spillage (the chow on the bottom of the cage) were weighed monthly to accurately measure the food consumption. All of the mice were weighed monthly. The mice were maintained pathogen-free in microisolator units on Tek FRESH® ultra laboratory bedding. Sentinel mice housed in the same room and exposed weekly to bedding collected from the cages of experimental mice were sacrificed on receipt and every six months thereafter for monitoring of viral antibodies (Mouse Level II Complete Antibody Profile CARB, Ectro, EDIM, GDVII, LCM, M. Ad-FL, M. Ad-K87, MCMV, MHV, M. pul., MPV, MVM, Polyoma, PVM, Reo, Sendai; BioReliance, Rockville, MD). All tests were negative.

Determination of Trx2 expression
The mitochondrial fraction obtained from several tissues (liver, kidney, brain) from young (4-6 months old) Tg(TXN2)−/0 x Trx1KO and WT mice were used to measure thioredoxin 2 (Trx2) levels by Western blot analysis with rabbit anti-Trx2 polyclonal antibody (Catalog No. LF-PA0012; LabFrontier, Seoul, South Korea) [4, 14]. After incubation with the primary antibodies, membranes were incubated with the respective peroxidase-linked secondary antibodies (Catalog No. P0217; Dako, Carpenteria, CA). Chemiluminescence was detected using the ECL Western blot detection kit (Amersham Biosciences Corp., Piscataway, NJ).

Trx1 levels
Trx1 levels were measured using cytosolic fractions obtained from tissues (liver, kidney, brain) of young (4-6 months old) Tg(TXN2)−/0 x Trx1KO and WT mice. Western blot analysis using goat anti-human Trx1 polyclonal antibodies (Catalog No. 705; American Diagnostica, Inc., Greenwich, CT) was performed as previously described [2, 3]. Total Trx1 (both oxidized and reduced forms) levels were measured by these antibodies. After incubation with the primary antibody, membranes were incubated with the peroxidase-linked secondary antibody (Catalog No. P0449; Dako, Carpenteria, CA). Chemiluminescence was detected with an ECL Western blot detection kit (Amer- sham Biosciences Corp., Piscataway, NJ).
Total glutathione levels

The Bioxytech GSH-420 kit (Catalog No. 21023; Oxis International, Inc., Foster City, CA) was used to measure the levels of total glutathione in several tissues (liver, kidney) from young (4-6 months old) Tg(TXN2)+/− x Trx1KO and WT mice.

Determination of major antioxidant enzyme activities: Cu/ZnSOD, MnSOD, glutathione peroxidase, and catalase

Major antioxidant enzymes [Cu/ZnSOD, MnSOD, glutathione peroxidase (GPx), and catalase] activities were measured in tissue homogenates obtained from the liver and kidney of young (4-6 months old) Tg(TXN2)+/− x Trx1KO and WT mice. The antioxidant enzymatic activity assays were conducted with the supernatants. GPx activity in tissue homogenates was measured by the assay as previously described [15]. The Catalase-520TM assay kit (OxisResearchTM, Portland, OR) was used to measure catalase activity by measuring the decomposition of hydrogen peroxide at 520 nm. MnSOD and Cu/ZnSOD levels were determined using activity gels [16, 17]. Images of the gels were analyzed by ImageQuant software.

Survival study

Mice in the survival groups were allowed to live out their lives, and the lifespan for individual mice was determined by recording the age of spontaneous death. A survival study was conducted with 35 Tg(TXN2)+/− x Trx1KO and 35 WT male mice. The survival curves comparison was statistically analyzed by the log-rank test [18]. The data for mean, 75th percentile, median, and 10th percentile (when 90% of the mice had died) survival were calculated for Tg(TXN2)+/− x Trx1KO and WT mice. The mean survivals for Tg(TXN2)+/− x Trx1KO and WT male mice were compared by performing a Student's t-test upon log-transformed survival times. The median and 10th percentile survivals for Tg(TXN2)+/− x Trx1KO and WT mice were compared using a score test adapted from Wang et al. [19].

Cross-sectional pathological assessment

Tg(TXN2)+/− x Trx1KO (n = 21) and WT (n = 40) male mice were assigned to the cross-sectional pathological analyses at 22-26 months of age. The gross pathological examinations to detect the visible tumors and other age-related lesions were conducted with each animal. Then, the following organs and tissues were excised and preserved in 10% buffered formalin: brain, pituitary gland, heart, lung, trachea, thymus, aorta, esophagus, stomach, small intestine, colon, liver, pancreas, spleen, kidneys, urinary bladder, reproductive system (prostate, testes, epididymis, and seminal vesicles), thyroid gland, adrenal glands, parathyroid glands, psoas muscle, knee joint, sternum, and vertebrae. Any other tissues with gross lesions were also excised. After the fixation, tissues were processed conventionally, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin-eosin. Histological classifications in aging mice were conducted after the diagnosis of each histopathological change was made [20, 21]. A list of pathological lesions for both neoplastic and non-neoplastic diseases was constructed for each mouse. These histopathological data were used to determine the tumor burden, disease burden, and severity of each lesion in each mouse [1-4, 21-23].

Statistical analysis

Unless otherwise specified, all experiments were done at least in triplicate. Data were expressed as means ± SEM and were analyzed by the non-parametric test ANOVA. All pair-wise contrasts were computed using Tukey error protection at 95% CI unless otherwise indicated. Differences were considered statistically significant at p < 0.05.

Results

Levels of Trx1 and Trx2 in tissues from Tg(TXN2)+/− x Trx1KO mice

The levels of Trx1 and Trx2 in tissues (liver, kidney, brain) from young (4-6 months old) Tg(TXN2)+/− x Trx1KO and WT mice were measured using Western blot analysis. The Trx2 protein levels were significantly higher (approximately 1.3- to 3-fold) in all three tissues examined in young Tg(TXN2)+/− x Trx1KO mice compared to
their WT littermates (Figure 1A; p < 0.05). Levels of Trx1 were significantly lower (approximately 50% less) in all three tissues examined in young Tg(TXN2)$^{+/0}$ x Trx1KO mice compared to their WT littermates (Figure 1B; p < 0.05).

Levels of total glutathione and major antioxidant enzymes in tissues from Tg(TXN2)$^{+/0}$ x Trx1KO mice

To examine whether the reduced Trx1 along with increased levels of Trx2 affects the levels of glutathione, which has similar biological functions to Trx, we measured total glutathione levels in liver and kidney from young (4-6 months old) Tg(TXN2)$^{+/0}$ x Trx1KO and WT mice. The data in Figure 2 show that levels of total glutathione in the tissues (liver, kidney) were similar between Tg(TXN2)$^{+/0}$ x Trx1KO and WT mice at 4-6 months (Figure 2; p > 0.05). Tg(TXN2)$^{+/0}$ x Trx1KO and WT mice showed similar activity/levels for major antioxidant enzymes (Cu/ZnSOD, MnSOD, GPx, and catalase) in the liver and kidney at 4-6 months (Figure 3; p > 0.05).

Survival curves, body and organ weights, and food consumption

The survival study was conducted with male Tg(TXN2)$^{+/0}$ x Trx1KO (n = 35) and WT (n = 35) mice (Figure 4). The survival curve of Tg(TXN2)$^{+/0}$ x Trx1KO mice was not significantly different from WT mice (Log-rank: p = 0.45). The mean, 75th percentile, median, and 10th percentile survival for 1) WT mice were 746, 470, 763, and 1,117 days; and 2) Tg(TXN2)$^{+/0}$ x Trx1KO mice were 835, 678, 791, and 1,141 days, respectively (Figure 4). Tg(TXN2)$^{+/0}$ x Trx1KO mice had slightly longer mean (10.7), 75th percentile (30.7%), median (3.5%), and 10th percentile (2.1%) lifespans compared to WT mice, although these differences were not statistically significant (p > 0.05). Tg(TXN2)$^{+/0}$ x Trx1KO mice had similar body and organ weights to WT mice at 4-6 months of age (Table 1). Tg(TXN2)$^{+/0}$ x Trx1KO and WT mice had similar food intake (data not shown).

Cross-sectional pathology

The cross-sectional pathological analyses were conducted with 21 Tg(TXN2)$^{+/0}$ x Trx1KO mice and 40 WT mice (22-26 months old). The major age-related pathology was a neoplastic disease, and commonly observed tumors were lymphoma, hemangiomia/hemangiosarcoma (liver and spleen), adenocarcinoma (lung), and hepatocellular carcinoma (liver), which is consistent with previous pathology results from the mice that have C57BL6 genetic background [1-4, 21]. The tumor burden, which is the total number of different types of tumors for each mouse, in both Tg(TXN2)$^{+/0}$ x Trx1KO and WT groups is presented in Figure 5A. The tumor burden of Tg(TXN2)$^{+/0}$ x Trx1KO mice was similar to WT mice (p > 0.05). The incidence of lymphoma was 9% less in Tg(TXN2)$^{+/0}$ x Trx1KO (76%) than WT (85%) mice, and severity of lymphoma was also similar between Tg(TXN2)$^{+/0}$ x Trx1KO (2.50) and WT (2.74) mice (Figure 5B; p > 0.05).

We also compared the severity of glomerulonephritis, a major non-neoplastic disease, in Tg(TXN2)$^{+/0}$ x Trx1KO and WT mice. The severity of glomerulonephritis was slightly higher in Tg(TXN2)$^{+/0}$ x Trx1KO (1.38) than WT (1.05) mice, although the difference was not statistically significant (Figure 5C; p > 0.05).

The disease burden (total number of histopathological changes in a body) is a good index of tissue and cell injury, which increases with age. Importantly, long-lived mice (e.g., calorie-restricted, Ames, and GHRKO mice) attenuate its age-related increase [20, 22, 23]. The disease burden was slightly less in Tg(TXN2)$^{+/0}$ x Trx1KO (3.67) than WT (4.15) mice. However, these differences are not statistically significant (Figure 5D; p > 0.05).

Discussion

Thioredoxin (Trx) was initially discovered in the early 1960s as the reductant for a variety of enzymes, and many studies demonstrated its unique roles in biology and physiology [6]. Trx is a hydrogen donor involved in reductive reactions of enzymes (e.g., ribonucleotide reductase, peroxiredoxin (Prx), and methionine sulfoxide (MetO) [7-12].
which play important roles in biology and cellular function. Trx also plays a key role in maintaining a reduced cellular environment, which provides protection against oxidative stress and control signaling pathways [24-27]. Trx mainly localizes in the cytosol (hTrx1) [28] and mitochondria (hTrx2) [29] in human cells. Studies with mice null for either Trx1 or Trx2 showed that these mice are embryonically lethal, which demonstrated that the presence of Trx1 and Trx2 is essential for mammalian cells/tissues [30, 31]. Because substantial evidence indicates Trx and thioredoxin interacting protein (Txnip) regulate essential cellular functions [6, 32], there is much scientific interest in how Trx could regulate mammalian aging.

Effects of overexpressed or downregulated Trx in the cytosol and/or mitochondria on aging and age-related diseases have been systemically investigated by our laboratory using transgenic and knockout mice for Trx1 or Trx2 [1-5, 21]. The first Trx study we conducted involved survival experiments with two lines of Trx1 transgenic mice [Tg(act-TrxN)\textsuperscript{+/-}] and Tg(TxN)\textsuperscript{+/-} mice] [2, 3]. These survival studies demonstrated that overexpression of Trx1 only showed a slight extension in the earlier part of life, and no significant effects on maximum lifespan were observed, possibly due to accelerated tumor development in the later part of life [2, 3].

The next study we conducted was a survival experiment with Trx2 transgenic mice [Tg(TxN)\textsuperscript{+/-}]. This study was conducted because of the strong indication that the protection of the mitochondria from age-related increases in oxidative stress may delay aging. The survival curve for Tg(TxN)\textsuperscript{+/-} mice showed a slight (approximately 8-9%) extension of mean, median, and 10th percentile lifespans compared to WT mice, although the extension of lifespan was not statistically significant. Tg(TxN)\textsuperscript{+/-} mice also showed a trend that the severity of lymphoma was slightly higher than WT mice [4].

These studies using Trx1 and Trx2 transgenic mice could indicate that overexpression of Trx in only one compartment of the cell may not have a significant impact on aging. Our studies with Trx1 or Trx2 knockout (KO) mice also showed down-regulation of Trx only in one compartment of the cell showed little impact on aging [5, 14]. Therefore, we decided to test the combined effects of Trx expression changes in both the cytosol and mitochondria. Among the possible combinations of changes (either up-regulation or down-regulation) of Trx1 and Trx2, we chose to down-regulate Trx1 and overexpress Trx2 in mitochondria for this study because one of the potential reasons Trx2 overexpression did not significantly extend lifespan could be due to its effects on age-related tumor formation [4]. Our previous study showed that Trx1 down-regulation (Trx1KO mice) had a subtle reduction of age-related tumor incidence [5]. Therefore, the purpose of this study is to test if reduced Trx1 along with Trx2 overexpression could extend the lifespan and attenuate tumor formation in Tg(TxN)\textsuperscript{+/-} x Trx1KO mice.

To test the effects of reduced Trx1 along with Trx2 overexpression on aging and age-related diseases, we generated Tg(TxN)\textsuperscript{+/-} x Trx1KO mice by crossing male heterozygous Trx1KO mice with female hemizygous Trx2 transgenic mice [Tg(TxN)\textsuperscript{+/-}]. These genetic manipulations did not cause changes in food intake, body weight, or organ weight. Young (4-6 months old) male Tg(TxN)\textsuperscript{+/-} x Trx1KO mice showed significantly higher Trx2 and significantly reduced Trx1 levels in all the tissues examined.
compared to WT control mice. The Trx2 and Trx1 levels were 1.5- to 3-fold higher and approximately 50% less in all of the tissues examined, respectively. The changes in Trx2 and Trx1 levels in male Tg(TXN2)KO x Trx1KO mice did not cause changes in total glutathione levels or the activities of major antioxidant enzymes (Cu/ZnSOD, MnSOD, GPx, and catalase). Our survival study demonstrated that the survival curve of male Tg(TXN2)KO x Trx1KO mice was not significantly different from WT control mice. Interestingly, the Tg(TXN2)KO x Trx1KO mice showed a slight extension of lifespan in the early stage of life, i.e., 75th percentile lifespan of Tg(TXN2)KO x Trx1KO mice was 30.7% longer than WT mice although these changes were not statistically significant. Mean, median, and 10th percentile lifespans of Tg(TXN2)KO x Trx1KO mice were similar to WT control mice. The cross-sectional pathology data showed that both Tg(TXN2)KO x Trx1KO and WT mice had a similar number of total tumors (tumor burden) [Tg(TXN2)KO x Trx1KO mice: 1.24; and WT control mice: 1.30]. The incidence of lymphoma was approximately 10% less in Tg(TXN2)KO x Trx1KO than WT control mice, however, the severity of lymphoma was similar between Tg(TXN2)KO x Trx1KO and WT mice. In addition to neoplastic diseases, the total number of pathological changes (disease burden) was also similar between Tg(TXN2)KO x Trx1KO and WT control mice. The severity of glomerulonephritis, which is one of the major non-neoplastic lesions, was slightly higher in Tg(TXN2)KO x Trx1KO than WT mice, however, this difference was not statistically significant (p > 0.05). The disease burden was slightly less in Tg(TXN2)KO x Trx1KO (3.67) than WT (4.15) mice, which also did not reach statistical significance.

Declarations

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Conflict of interest: The author declares no conflict of interest.

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