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The Transcription of Liver Thioredoxin Following the Ionizing Irradiation of Radioresistant and Radiosensitive Mice

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The radiation protective effect of thioredoxin (TRX) in a bacterial system has been reported and based upon this observation we were interested to examine TRX transcription in the mammalian system following ionizing irradiation. In order to answer the question whether radiation sensitive mice (BALB/c) showed TRX transcription different from radiation resistant mice (C3H), we exposed these strains to X-ray doses of 2 Gy, 4 Gy and 6 Gy. Groups consisting of 6 mice were sacrificed 5, 15 and 30 minutes after irradiation and livers were immediately taken into liquid nitrogen. Total RNA was isolated from the organs by the use of a commercially available kit and used for Northern blots and slot blots with a chemiluminescence technique. Northern blots revealed a single band at 538 bp for TRX and at 1.8 kb for beta-actin. Quantification of mRNA TRX by densitometry of slot blots revealed that C3H transcribed TRX significantly higher at an earlier time point (5 min) than BALB/c. This delayed transcription of TRX in the radiosensitive mouse strain showed a comparable pattern at three different radiation doses and may well be responsible for radioresistance although no quantitative differences of TRX transcription between BALB/c and C3H mice were detectable.

Keywords: Thioredoxin, mRNA, radiation resistance, mice, ionizing irradiation, X-ray, transcription, beta-actin

INTRODUCTION

Thioredoxin is a low molecular weight protein abundant in cells.[1] TRX is transferring reducing equivalents to a series of enzyme systems as e.g. methionine sulphoxide reductase[2] and ribonucleotide reductase.[3] Although a bulk of physiological functions have been assigned to TRX, mutants lacking TRX showed no abnormalities of growth or function.[4] Based upon the shown hydrogen donation TRX was tested for it's radiation protective function in bacteria: Bacterial strains, engineered to express different TRX levels, were used in these experiments and cells grown to late stationary phase demonstrated a decreasing sensitivity to gamma radiation paralleling increasing TRX. Exponentially growing bacteria, however, were equally sensitive to ionizing irradiation, regardless of intracellular TRX. The authors also showed that cells of the radiation resistant phenotype in the stationary phase

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reverted to the radiation sensitive phenotype when diluted into fresh growth medium.[5] As mechanisms of action they proposed firstly the reaction of TRX with radical species, secondly, the interaction of TRX with a radical induced on a critical target molecule and thirdly, that TRX may activate DNA repair, a mechanism shown for other thiol compounds.[6,7,8] We were interested whether TRX transcription following ionizing irradiation in the mammalian system would be increased. In addition, we investigated whether transcriptional differences between radiationsensitive (RS) and radiationresistant (RR) mice would be found.

MATERIALS AND METHODS

Animals and Irradiation Protocol

60 female BALB/c/JHim (RS) and 60 female C3H/HeHim (RR), mean age 90 ± 3 days, were used in the experiments. Their differences in the radiobiological response are well-known and documented.[9] All experiments were carried out with the permission of the German Committee on Animal Experiments (Bonn). Animals were divided into a RS and RR control group, and the other six (three RS and three RR) groups were irradiated with 2, 4 and 6 Gy. Mice were sacrificed by neck dislocation after 5, 15 and 30 min. Each group consisted of six animals. Whole body irradiation was performed with a X-ray tube 200 RT (C.H.F. Mueller, Germany). 200kV, 20 mA, 0.1 mm Cu filters and a dose rate of 2 Gy per min were applied.

mRNA Isolation from Mouse Liver, Preparation of Probes, Northern Blots and Slot Blots

The organs were obtained at autopsy and taken immediately into liquid nitrogen. Frozen liver samples were ground and total RNA extraction was performed using the RNeasy minipreps extraction kit (QUIAGEN). Subsequently, total RNA was applied onto 1.4% agarose gel after denaturation with glyoxal and dimethylsulfoxide according to the method by McMaster and Carmichael[10] and electrophoresed at 3–4 V/cm for 2.5 hrs in circulating 0.01M phosphate buffer pH 7.0. RNA was then transfered to a positively charged nylon membrane (Hybond N+, Dupont, NEF 986) by capillary blotting[11] and fixed with 0.05 N sodium hydroxide for 5 min at room temperature and finally equilibrated at pH 7.0 with 3 washes in 2XSSC.

Probes for human beta-actin (ATCC 9800, human beta actin cDNA probe) and Thioredoxin (plasmid pcdsr alpha 7-2, from Prof Yodoi Junji and Prof. Matsui Minoru, Dpt Biological Responses, Kyoto University, Japan,) were used for Northern and slot blots.

For transfection an aliquot of frozen competent cells (E. coli HB 101) was thawed to O°C and 5 ul of the plasmid (5 ug/100 ul) were incubated with the competent cells on ice, followed by an incubation step at 90 sec at 42°C and returning the tubes to the ice bath for 2 min. Subsequently, 1 ml of prewarmed (37°C) SOC medium were added and incubated for 60 min on a shaker.

Then tubes were centrifuged for 10 min at 4500 × g, the pellet resuspended in LB medium at 3 different dilutions and plated in LB Agar medium plus ampicillin (100 ug/ml) where they were allowed to grow over night at 37°C.

A single bacterial colony was transferred to 4 ml LB medium containing ampicillin and the culture was incubated overnight at 37°C with vigorous shaking 1 ml of the tube was inoculated in 500 ml of LB medium containing ampicillin prewarmed to 37°C in a 2 l flask; this culture was incubated under vigorous shaking until the OD at 600nm was 0.4.

For the isolation of the pcDSR alpha 7-2 plasmid the Plasmid Maxi kit (Omega 12145), based upon a modified alkaline lysis procedure, was used followed by the binding of plasmid DNA to an anion-exchange resin under appropriate low salt and pH conditions. Plasmid DNA is eluted

by this principle by a high salt buffer and concentrated and delated by isopropanol precipitation.

For the digestion with restriction enzymes 3 ug of plasmid, 2 ul SuRECut buffers for restriction endonucleases (Boehringer Mannheim), 2 ul BAM Hl, 2 ul ECO R1 (Boehringer Mannheim), 9 ul water were incubated during 3 hrs in a thermoblock at 37°C. After the reaction was stopped the solution was electrophoresed on 1% agarose gel in 0.15 M Tris borate buffer pH 8.0 in order to show the length of fragments. Single bands for TRX and for beta-actin were shown.

Using QUIAquick gel extraction (QUIAGEN 28704), a method with selective DNA binding properties of a silica gel membrane, the DNA probe was isolated and quantified at 260 nm.

The probe was denatured prior to labelling by boiling for 5 min and subsequent cooling on ice and labeled with fluorescein-12-dUTP using the Renaissance Random Primer Fluorescein-12 d-UTP labeling kit (Dupont, NEL 203).

After fixation of bound RNA, the nylon membrane was incubated in pre-hybridization solution (0.25M phosphate buffer pH 7.2, containing 5% SDS w/v, 1 mM EDTA and 0.5% blocking reagent [from Dupont NEL 203]) for 12 hrs at 65°C in a hybridization oven. The blots were hybridized overnight at 65°C with the labelled probes each (50 ng/ml of pre-hybridization buffer).

After hybridization, nonspecifically bound material was removed by post-hybridization washes with 0.5× and 0.1× pre-hybridization buffer 2 × 10 min each at 65°C. The 0.5× and 0.1× pre-hybridization buffer was brought up to 65°C prior to use and the second wash was performed at room temperature.

Hybridized blots were blocked with 0.5% blocking reagent in 0.1M Tris HCl pH 7.2 and 0.15 M NaCl for 1 hr at room temperature. Membranes were then incubated with antifluorescein HRP antibody (Dupont NEL 203) at a 1:1000 dilution in the solution given above for 1 hr under constant shaking.

Membranes were washed 4× for 5 min each in the solution given above.

The Nucleic Acid Chemiluminescence Reagent (Dupont NEL 201) was added to the membranes and incubated for 1 min. Excess detection reagent was removed by the used of filter paper, the membrane was placed in Sarawrap paper and exposed to autoradiography Reflection films (Dupont NEF 496) for 15 min at room temperature.

Slot blots were performed according to the method of White and Bancroft.[12] This procedure consisted of placing 2 ug of total RNA dissolved in 10 ul double distilled water mixed with 500 ul of 100% formamide, 162 ul of 37% formaldehyde, 100ul of 10× MOPS buffer. This mixture was incubated for 10 min at 65° C and cooled down subsequently on ice. Samples were placed onto the membrane by the Manifolds filtration equipment (slot blot apparatus Bio slot TM, BIORAD) and the hybridization was performed as described above.

Denistometry of films was performed using the Hirschmann elscript 400 densitometer (Germany).

Statistical Method

The ANOVA with subsequent Kruskal Wallis Test and Student's t test was applied. A significant differences was considered at the $p < 0.05$ level.

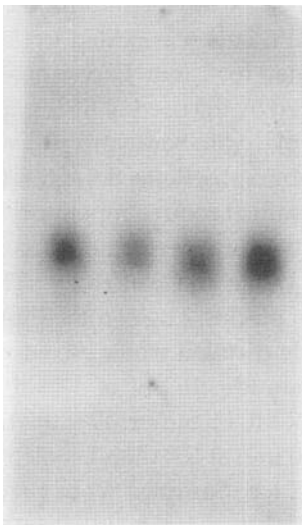
RESULTS

The Northern blot pattern of beta-actin and TRX hybridization revealed a single band at 538 bp for thioredoxin and at 1.8 kb for beta-actin and is given in Figure 1.

Table I shows means and SEM of TRX mRNA/beta-actin mRNA.

No quantitative difference of transcription in terms of TRX mRNA/beta-actin mRNA was found between the unirradiated RR and RS mice.

2 Gy experiments: At five minutes RR mice showed a significantly increased TRX transcription which decreased to background levels within 30 min. The RS mice showed significantly elevated transcription later, i.e. at 15 min, declining to background levels at 30 min.



a)

b)

FIGURE 1 **a**) Northern blot revealing a single band at 538 bp characterizing the specific reaction with the probe for TRX in unirradiated RS and RR mice (lane 1,2; left to right) and 6 Gy irradiated mouse strains (lane 3,4). **b**) Northern blot revealing a single band at 1.8 kb characterizing the specific reaction with the probe for the housekeeping gene beta-actin in unirradiated RS and RR mice (lane 1,2; left to right) and 6 Gy irradiated mouse strains (lane 3,4).

4 Gy experiments resembled the pattern obtained at 2 Gy: As early as at 5 min following irradiation a significantly higher TRX transcription was found in the RR group, which declined to background levels from 15 min. RS animals increased TRX transcription not before 15 minutes, again returning to background levels at 30 min.

6 Gy experiments showed a comparable pattern of TRX transcription: RR mice transcribed TRX as soon as at 5 min with a rapid decline from 15 min. RS mice showing background levels at 5 min following irradiation, significantly increased TRX transcription as late as 15 min following irradiation and a rapid decline at 30 minutes.

Statistical determinations did not showed significant differences comparing the RR with the RS group.

DISCUSSION

Our results clearly demonstrate that the transcription of TRX in the RR mice takes place earlier than in RS mice at all radiation doses evaluated. The underlying mechanism (s) for the time differences of TRX transcription remains unclear and whether specific or unspecific transcription factors may be involved is speculation. Delayed TRX transcription would be compatible with a role of TRX for radioresistance. No quantitative transcriptional differences were observed, which is not a contradiction to the observation of a radiation protection effect by TRX, however. Our results are therefore compatible with the findings described by Lunn and Pigiet[5] who reported radiation protection against ionizing radiation by TRX at the protein level, suggesting scavenging activity of active oxygen species by TRX, a "high molecular weight thiol". It also may exert its radiation protective role by the transfer of reducing equivalents as described for high and low molecular weight thiols.[13,14] Thus transfer of reducing equivalents seems to play a certain radiation protective role, which is not unequivocal, however. The use of low molecular weight thiols with hydrogen donation as the theoretical mechanism of action is still holding centre stage.[15,16]

It is still an open question whether TRX and other antioxidant proteins and enzymes as metal-

TABLE I Means and SEM of mRNA TRX/mRNA β-actin

			5min	15min.	30min.
Unirradiated	C3H	1,28 ± 0,56			
	BALB/c	1,50 ± 0,86			
2 Gy	C3H		2.19 ± 0.25	1,25 ± 0,21	1,02 ± 0,51
	BALB/c		0.85 ± 0.50	3,21 ± 1,13	1,10 ± 0,49
4 Gy	C3H		2.81 ± 1.60	1,08 ± 0,26	1,29 ± 0,40
	BALB/c		1.02 ± 0.18	1,92 ± 0,30	1,19 ± 0,32
6 Gy	C3H		2,27 ± 0.76	0,75 ± 0,15	1,07 ± 0,18
	BALB/c		1.04 ± 0.26	2,68 ± 1,34	0,99 ± 0,22

lothioneins,[17,18] superoxide dismutase,[19] catalase, glutathion peroxidase and -transferase[20] significantly contribute to radiation resistance. With the increasing use of molecular biological methods and the availability of probes for antioxidant enzymes, the case is being re-evaluated as regulation mechanisms seem to be studied reliably at the transcriptional level. In addition, the promising studies on DNA repair and radiation resistance were put into the center and the use of repair deficient rodent cells is considered a specific approach to the solution of this question and clearly separates radiation resistant from radiation sensitive at the level of DNA repair. A gene named XRCC1 (X-ray—repair cross complementing) has been shown to cross complement radiation sensitive cell lines making them radiation resistant.[21] These interesting findings, however, should not lead to the suggestion that studies on the antioxidant systems, particularly at the transcriptional level, were obsolet.

The C3H mouse is considered as radiation resistant[9] but the quantitative transcription of TRX was not only similar before and after irradiation but also comparable to the TRX mRNA levels shown by the radiation sensitive BALB/c mouse, both pre- and post irradiation, read at different radiation doses at different time points. Therefore, TRX transcription in quantitative terms should not be responsible for the difference in genetic susceptibility to ionizing radiation. Our findings of a *delayed transcriptional response* to irradiation, however, may well be indicating a role in the different radioresistance observed in the two mouse strains. Our studies at the transcriptional level provide a possible regulation mechanism but warrant and challenge studies at the protein level.

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