ORIGINAL RESEARCH

Overexpression of CIRP may reduce testicular damage induced by cryptorchidism

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Abstract

Purpose: To investigate the protective effect of overexpression of cold-inducible RNA-binding protein (CIRP) on testicular damage induced by cryptorchidism.

Methods: Male BALB/c mice were made surgically cryptorchid and CIRP gene was transferred into the cryptorchid testis by *in vivo* electroporation. Seven or ten days after electroporation, the expression of CIRP, p53 and Fas mRNA and protein were analyzed by reverse-transcription polymerase chain reaction (RT-PCR) and immunoblotting, respectively. Meanwhile, Histopathological changes were observed by light microscope, and flow cytometry was used to detect testicular cell apoptosis.

Results: Testicular weights after transfection with pVAX1-CIRP or pVAX1 were 0.083 ± 0.005 g and 0.065 ± 0.004 g, respectively, on day 7 ($P < 0.05$) and 0.078 ± 0.004 g and 0.052 ± 0.007 g, on day 10 ($P < 0.05$). Testicular cell apoptosis after transfection with pVAX1-CIRP or pVAX1 were 9.8 ± 1.1 % and 20.7 ± 1.3 %, respectively, on day 7 ($P < 0.01$) and 10.4 ± 0.9 % and 27.5 ± 1.2 %, on day 10 ($P < 0.01$). In addition, the expression of CIRP mRNA and protein in the testes transfected with pVAX1-CIRP were both increased ($P < 0.05$) at each indicated time point. Meanwhile, the expression of p53 was decreased on day 7 ($P < 0.05$) and Fas was decreased on day 10 ($P < 0.05$).

Conclusions: Overexpression of CIRP may reduce testicular damage induced by cryptorchidism by down-regulating the levels of p53 and Fas.

Physiological scrotal hypothermia is necessary for normal spermatogenesis and fertility in mammals. In experimental animals, surgical induction of cryptorchidism or exposure to heat stress causes disruption of spermatogenesis, leading to infertility.^{1, 2} In the mouse, the effects of scrotal heating on the developing germ cells include altering synthesis of DNA, RNA and proteins, as well as protein denaturation and abnormal chromatin packing.^{3, 4}

CIRP is a recently identified cold-inducible RNA-binding protein that is inducible at 32°C in mouse somatic cells *in vitro*.⁵ It is a member of RNA-binding protein family that possesses an amino-terminal consensus-sequence RNA-binding domain (CS-RBD) and a carboxyl-terminal glycine-rich domain.⁶ It is structurally highly similar to YRRM1 and RBM1, candidates for the human azoospermia factor.^{7, 8} In the mouse testis, CIRP is constitutively expressed in the germ cells. When mouse testis was exposed to heat stress by experimental cryptorchidism or immersion of the lower abdomen in warm (42°C) water, CIRP expression was decreased in the testis. In human testis with varicocele, the expression of CIRP was decreased compared with that in nonvaricocele patients.⁹ As the timing of mitosis and meiosis in spermatogenesis is

strictly controlled, decreased expression of CIRP may adversely affect their coordinate regulation, and lead to disruption of spermatogenesis and apoptosis at 37°C.¹⁰

The tumor suppressor protein, p53, is highly expressed in the testis^{11,12} and is known to be involved in apoptosis.^{13, 14} Studies have indicated that p53 plays an important role in temperature mediated germ cell loss.^{15,16} Besides, Fas and Fas ligand (FasL) interaction is a major apoptotic mechanism found in various tissues and cell types. In the mammalian testis, Fas is expressed in germ cells and FasL is detected in Sertoli cells.^{17,18} Testicular germ cells are susceptible to Fas antibody-induced apoptosis.¹⁹

In this study, we try to enhance the level of CIRP in cryptorchidism and observe whether CIRP overexpression could protect cryptorchid testes from impairment induced by heat stress. In addition, we investigated the relation between CIRP and the levels of p53 and Fas.

Materials and methods

Construction of Plasmid vector

Plasmid pVAX1-CIRP was constructed by inserting mouse CIRP cDNA (a gift from Dr J. Fujita of the Department of Clinical Molecular Biology, Kyoto University, Japan) into pVAX1 expression vector (Invitrogen, USA) containing the CMV promoter. The empty pVAX1 was used as a control. DNA was diluted in phosphate-buffered saline (PBS) to 1.0 µg/µl immediately before injection.

Animal

Forty-eight male BALB/c mice (7-wk-old) were purchased from the Experimental Animal Center of Wuhan University (Wuhan, China). Animal procedures used in this study conformed to NIH guidelines and was approved by the Ethical Committee, Wuhan University. The mice were housed in a temperature

controlled room (24°± 1°C) under a 12-hour light-dark cycle, with free access to food and water.

Surgical procedures

Each mouse was made surgically cryptorchid. Under general anesthesia (sodium pentobarbital), the left testis was surgically exposed when a mid-line incision was made, gubernaculum was cut and then the testis was exposed under a dissecting microscope. A small (2–3-mm) incision was made in the tunica, then 20 µl per testis of plasmid DNA of pVAX1-CIRP (pVAX1-CIRP group) or pVAX1 (pVAX1 group) was injected into seminiferous tubules with a micro injector and injections were made at three sites in each testis according to the procedure of Yamazaki Y et al.²⁰ The mice in the PBS group were injected with 20 µl of PBS containing no DNA, aiming to evaluate the likelihood of testicular damage. Immediately after the injection, three electric pulses of 30 V each were delivered to each injection site, each pulse lasting 20 ms. Then, the testis was fixed to the lateral abdominal wall by suture and the skin was stitched. Seven days or 10 days after the electroporation, eight testes taken from each of the three groups were examined. The tissues were fixed with 4% polyoxymethylene and processed for routine hematoxylin and eosin (H&E) staining.

Flow cytometry

Annexin V-FITC and propidium iodide (PI) (BD Pharmingen) double staining were carried out to discriminate and quantify cell death by apoptosis or necrosis. Fifty micrograms of testicular tissue were made into cell suspension, adjusting the cell density to 1×10⁶/ml. After the cells were resuspended twice in 100µl binding buffer, 10ul Annexin V-FITC was added with gentle mixing and the cells were incubated in the dark for 15 min. Then, the labeled cells were added to 5 µl PI in 300 µl binding buffer with gentle mixing before immediate analysis with a FACScan flow cytometer (Becton–Dickinson, UK). A total of 10,000 cells were analyzed per sample. Data were re-

TABLE 1. List of the primer sets used in the present experiment

Genes	Forward (F) and reverse (R) sequences	Product sizes (bp)
CIRP	F: 5'-CCGAGGCTTTGGGTTTGT-3' R: 5'-ATAGCTGCCACCCTGACT-3'	316
P53	F: 5'-TCTGGGACAGCCAAGTCTGT-3' R: 5'-GGAGTCTTCCAGTGTGATGA-3'	435
Fas	F: 5'-GACTGCAAAATGAATGGGGGT-3' R: 5'-AGTGTCTGGGGTTGATTTTC-3'	750
β -actin	F: 5'-GGGCTG TATCCCCTCCATC-3' R: 5'-GTCACGCACGATTCCCTCTC-3'	552

corded and processed using the CellQuest software (Becton–Dickinson, UK).

RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, CA, USA) and cDNA was synthesized using M-MuLV reverse transcriptase and random primers (Fermentas, USA). Amplification of cDNA by PCR using specific primer pairs for CIRP, p53 and Fas (Table 1) was performed in 50 μ l reaction volumes containing 50 mM KCl, 10 mM Tris-HCl, pH 8.8, 1.5 mM MgCl₂, 0.08% Nonidet P40, 0.2 mM of each dNTP, 0.2 μ M of each primer, 1.25 unit of Taq DNA polymerase (Fermentas, USA) and 2.0 μ l cDNA as template. PCR conditions were: 30 sec at 95°C, 45 sec at 55°C, and 45 sec at 72°C for the indicated number of cycles (30cycles for CIRP, 25cycles for Fas, p53 and β -actin). PCR products were separated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide (Sigma, USA). The relative expressions of interesting genes were semiquantitatively compared with β -actin gene expression using an image analysis instrument.

Immunoblotting

Total protein was extracted using RIPA Lysis buffer with protease inhibitors and protein concentration was determined using a BCA kit (Pierce, IL, USA). Fifty micrograms of protein extract were separated using 15% SDS-PAGE, electro-transferred to a PVDF membrane and blocked using 5% non-fat milk in Tris-buffered saline with 0.1% Tween 20 (TBST). After

blocking, membranes were washed three times with TBST and then respectively incubated with an anti-CIRP antibody (ProteinTech, IL, USA), an anti-p53 antibody (bioss, China), an anti-Fas antibody (bioss, China) and an anti-GAPDH antibody (Santa Cruse, USA) in TBST. Following incubation in the primary antibody, membranes were washed three times with TBST, incubated with horseradish peroxidase-conjugated secondary antibodies (ProteinTech, IL, USA) in TBST and then washed again before visualized with a diaminobenzidine (DAB) reagent kit (Zsbio, China). GAPDH was used as a protein loading control.

Statistical analysis

SPSS (version 11.5) statistical package (SPSS, Chicago, IL, USA) was used for the statistical analysis. Numerical values were expressed as the mean $\pm$ SD. Statistical analysis was carried out using one-way ANOVA followed by the Student-Newman-Keuls method for multiple comparisons among means. $P < 0.05$ was considered statistically significant.

Results

Testicular weight and evaluation of spermatogenesis

The weight of testis transfected with pVAX1-CIRP was heavier than that in pVAX1 or PBS-treated mice ($P < 0.05$). Testicular weight was not different between pVAX1 and PBS-treated mice ($P > 0.05$), but testicular weight on day 10 was much lighter than that on day 7 in pVAX1 or PBS-treated mice ($P < 0.05$). No difference of testicular weight was noted at different time

TABLE 2. Testicular weight and testicular cell apoptotic rates

Groups	n	Testicular weight(g)		Apoptotic rate (%)	
		day 7	day 10	day 7	day 10
pVAX1-CIRP	8	0.083±0.005 [#]	0.078±0.004 [#]	9.8±1.1 [*]	10.4±0.9 [*]
pVAX1	8	0.065±0.004 [△]	0.052±0.007	20.7±1.3 [△]	27.5±1.2
PBS	8	0.063±0.006 [△]	0.049±0.004	21.6±1.4 [△]	28.3±1.1

[#]P<0.05, ^{*}P<0.01, compared with pVAX1 or PBS group

[△]P<0.05, compared with day 10

points when transfected with pVAX1-CIRP ($P>0.05$) (Table 2).

Evaluation of spermatogenesis by histological observation (HE staining)

The earliest cellular damages were noticed in primary spermatocytes and early spermatids. Testes treated with pVAX1 or PBS exhibited disordered and sloughed germinal cells, and the situation on day 10 was more serious than on day 7. Testes transfected with pVAX1-CIRP showed active spermatogenesis and orderly arrangement of germinal cells, and the situation on day 10 was similar to that on day 7 (Fig. 1).

Flow cytometry detection of apoptosis

Testicular cell apoptotic rate in each group is shown in Table 2. Testicular cells apoptotic rate was not different between pVAX1 and PBS-treated mice at each indicated time point, but the apoptotic rate on day 10 was much higher than on day 7 ($P<0.05$). Testicular cell apoptosis was decreased after transfecting with pVAX1-CIRP ($P<0.01$), and apoptotic rate on day 10 was similar to that on day 7.

RT-PCR detection of CIRP, p53 and Fas mRNA

The CIRP mRNA level in testes transfected with pVAX1-CIRP was higher than that in pVAX1 or PBS-treated mice at each indicated time point ($P<0.05$). Seven days after electroporation, p53 mRNA level in the testis transfected with pVAX1-CIRP was much lower than that in pVAX1 or PBS-treated mice ($P<0.05$), but Fas mRNA level was not different

among groups. Ten days after electroporation, the Fas mRNA level in testes transfected with pVAX1-CIRP was much lower than that in pVAX1 or PBS-treated mice ($P<0.05$), while p53 mRNA level was similar to that on day 7: β -actin mRNA was detected equally in all the groups (Fig. 2).

Immunoblotting detection of CIRP, p53 and Fas protein

Seven days after electroporation, p53 protein level in testies transfected with pVAX1-CIRP was much lower than that in pVAX1 or PBS-treated mice ($P<0.05$), while Fas protein level was not different among groups. Ten days after electroporation, Fas protein level in testes transfected with pVAX1-CIRP was much lower than that in pVAX1 or PBS-treated mice ($P<0.05$), while p53 protein level was similar to that on day 7. At each indicated time point, CIRP protein level in the testis transfected with pVAX1-CIRP was always higher than that in pVAX1 or PBS-treated mice ($P<0.05$). GAPDH protein was detected equal in all groups (Fig.3).

Discussion

In vivo electroporation has been shown to be an efficient method of transferring genes to the tissues of living animals.²¹ The combination of DNA injection into seminiferous tubules and subsequent in vivo electroporation resulted in an efficient and convenient assay system for gene expression during spermatogenesis²⁰. In the present study, we found that the expression of CIRP mRNA and protein in the testis transfected with pVAX1-CIRP were both increased at

FIGURE 1. Histopathological changes of mouse testes in different groups observed by light microscope (Hematoxylin and eosin staining, original magnification $\times 200$). (A) 7 days and (D) 10 days after testes were transfected with pVAX1-CIRP. (B) 7 days and (E) 10 days after testes were transfected with pVAX1. (C) 7 days and (F) 10 days after testes were treated with PBS. Bars=50 μ m.

each indicated time point. Therefore, we demonstrated that CIRP gene could be successfully transferred into mouse testis by in vivo electroporation.

A previous study demonstrated that abdominal heat stress induced cell apoptosis in the adult mouse testis beginning on day 6 of experimental cryptorchidism, with germ cell loss (demonstrated histologically and by reduction in testicular weight) on day 7, abdominal heat stress caused germ-cell loss by apoptosis in the adult testis.²² Similarly, in the present study, the weight of testes transfected with pVAX1 or PBS-treated mice had decreased by the 7th day after the induction of cryptorchidism, while the weight of testes transfected with pVAX1-CIRP was much greater. Besides, we found that testicular cell apoptosis in the testis transfected with pVAX1-CIRP was less than that in pVAX1 or PBS-treated mice. This suggests that CIRP could reduce the risk of germ cell loss in cryp-

torchidism by diminish testicular cell apoptosis induced by heat stress in cryptorchidism.

In studies of other cells, culture of mouse BALB/3T3 cells at 32 °C may protect them from a variety of stress conditions including DNA damage, endoplasmic reticulum stress, high osmolarity, and H₂O₂ by p53-dependent and p53-independent mechanisms.²³ Also, CIRP contributes to the anti-apoptotic effects of hypothermia through ERK signaling.²⁴

Recently, it has been shown that abdominal heat stress induces germ cell loss through two apoptotic pathways: p53 is responsible for the initial phase of germ cell apoptosis, and Fas is responsible for the second phase of apoptosis starting on day 10 of cryptorchidism.^{25, 26} In this study, we found that the expression of p53 on day 7 and Fas on day 10 were not increased in testes transfected with pVAX1-CIRP. This suggests that CIRP could down-regulate the lev-

FIGURE 2. (A) At each time point after electroporation, the expression levels of CIRP, p53 and Fas in mouse testes were detected by RT-PCR. Amplification of β -actin was used as an internal control. (B) Graphical representation of data shown in A. The densities of CIRP, p53 and Fas bands were compared with those of β -actin control, and the results were expressed as density ratios. * $P < 0.05$.

FIGURE 3. (A) At the indicated time points, mouse testes lysates were prepared and subjected to immunoblotting with indicated antibodies after the samples were separated by 15% SDS-PAGE. GAPDH was shown as a loading control. (B) Graphical representation of data shown in A. The densities of CIRP, p53 and Fas bands were compared with those of GAPDH control, and the results were expressed as density ratios. * $P < 0.05$.

els of p53 and Fas, which are major causes of testicular cell apoptosis in cryptorchidism.

In conclusion, we demonstrated that the CIRP gene could be transferred into the mouse testis by in vivo electroporation, and overexpression of CIRP may, to some extent, reduce testicular damage induced by heat stress in cryptorchidism. The mechanisms involved may be that CIRP can down-regulate the levels of p53 and Fas. These findings may generate a novel therapeutic strategy in cryptorchidism. However, we were uncertain that CIRP gene was transfected into Sertoli cells or germ cells in this study. Besides, there exist other unknown heat-induced apoptotic pathways in the testis.²⁶ Whether CIRP contributes to the protective effects against other apoptotic factors remains to be determined.

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