The Rb1 gene inhibits the viability of retinoblastoma cells by regulating homologous recombination

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Abstract. Retinoblastoma is a childhood ocular tumor caused by the inactivation of both alleles of the retinoblastoma gene (Rb1). Without Rb1 gene function, chromosomal aberrations are observed in retinoblastoma cells. The instability of the genome is closely associated with the repair of DNA double-strand breaks (DSBs). However, the precise molecular mechanism of action of Rb1 in DNA DSB repair remains unclear. Thus, in this study, we aimed to investigate whether the Rb1 gene affects DNA stability by assaying DNA DSB repair and also whether it regulates the proliferation of retinoblastoma cells. Rb1 immunofluorescence and RT-PCR were performed, demonstrating that the Rb1 gene is silenced in SO-Rb50 retinoblastoma cells, and the karyotype analysis of SO-Rb50 cells indicated that the loss of Rb1 function led to genomic instability; both numerical and structural chromosomal aberrations were observed in our study. In addition, the DNA DSB repair efficiency of the SO-Rb50 cells was measured by γ-H2AX immunofluorescence, a commonly used in situ marker of DNA DSBs, following exposure to ionizing radiation (IR) (2.5 and 5.0 Gy). We found that the DNA repair efficiency was significantly increased following IR-induced damage (P<0.01). However, there was no significant difference in DNA repair efficiency between the cells expressing exogenous Rb1 and the control (P>0.05). The assay for the screening of the effect of Rb1 on the sub-pathway of DNA DSB repair, non-homologous end joining (NHEJ) and homologous recombination (HR), indicated that Rb1 did not affect NHEJ activity, although it significantly promoted the HR pathway (HR levels increased by 2.46-fold) compared with the control (P<0.01). Furthermore, we found that the cell viability of the SO-Rb50 cells transfected with exogenous Rb1 was significantly inhibited (P<0.01) and cell cycle assay indicated that exogenous Rb1 induced S phase arrest (P<0.001) which also inhibited the proliferation of retinoblastoma cells (SO-Rb50) in vitro. Therefore, this study provides new insight into the mechanisms of action of the Rb1 gene in regulating the proliferation of retinoblastoma cells.

Introduction

Retinoblastoma is the most common ocular tumor classically initiated by the loss or mutation of both alleles of the retinoblastoma gene (Rb1) during retinal development. It is a pediatric tumor of the retina observed in approximately 1:15,000 live births (1). The current treatment of retinoblastoma includes chemotherapy, radioactive plaque, external beam radiotherapy, cryotherapy and surgery (2-4). Although the current survival rates of retinoblastoma exceed 90%, complications and side-effects exist, such as severe visual impairment or the loss of one or both eyes.

Previous studies on Rb1 have shown that the mutation on chromosome 13q is often present in retinoblastoma tumors. Rb1, a tumor suppressor gene, plays crucial role in the regulation of the cell cycle, cell differentiation, cell aging, apoptosis and growth suppression (5-7). Rb1 can be functionally inactivated through a variety of mechanisms, including deregulated phosphorylation and direct sequestration by oncoproteins. Evidence supports the notion that the loss of Rb1 function leads to a breakdown in genome integrity (8). According to previous studies, SO-Rb50, an Rb1-deficient cell line, displays obvious chromosomal instability. The chromosomal aberrations increase during long-term culture in vitro (9,10).

The E2F family of transcription factors plays a pivotal role in the regulation of cell cycle progression, DNA repair and replication, apoptosis, differentiation and development (11). E2F1, best known as the founding member of the E2F transcription factor family, has been implicated in the response to DNA damage in conjunction with retinoblastoma family proteins. The Rb1-E2F1 complex is formed in response to DNA damage and is recruited to the sites of DNA double-strand breaks (DSBs). Certain studies have suggested that E2F1 plays a crucial role in DNA DSB repair by promoting the recruitment and/or retention of repair factors, such as XPA and XPC, at the sites of DNA breaks (12,13). However, other studies have...
indicated that the loss of Rb1 has no significant effects on DNA DSBs, as shown by γ-H2AX foci intensity in the cells following exposure to ionizing radiation (IR) (14). Therefore, the precise molecular mechanism of action of Rb1 in chromosomal instability remains unclear.

Two distinct pathways have been described which ensure that DNA DSBs are repaired: DNA non-homologous end joining (NHEJ) and homologous recombination (HR). During HR, the damaged chromosome interacts via synopsis with an undamaged DNA molecule with which it shares extensive sequence homology, usually its sister chromatid (15,16). HR is most active in the late S and G2 phase of the cell cycle. By contrast, NHEJ is active throughout the cell cycle and requires little or no DNA homology during repair (17,18). The NHEJ pathway plays a key role in the repair of DNA DSBs caused by IR.

Based on the evidence of chromosomal instability in Rb1-deficient cells, as well as no evidence of any significant effects on DNA DSB repair in Rb1-deficient and wild-type (WT) cells following exposure to IR, we hypothesized that Rb1 plays a differential role in the sub-pathways of DNA DSB repair, NHEJ and HR. In order to confirm this hypothesis, we evaluated the pathway of Rb1-mediated DSB repair in retinoblastoma cells. We found that Rb1 significantly promoted HR, had no effect on NHEJ in retinoblastoma cells. This study provides new insight into the mechanisms of action of the Rb1 gene in the chromosomal instability of retinoblastoma cells.

Materials and methods

Plasmid construction. For pcDNA3.1-Rb1, WT Rb1 cDNA was inserted into the pcDNA3.1-Myc-His vector between the KpnI and NotI restriction sites. The pEGFP-HR plasmid as a substrate for recombination was derived from pEGFP-N1 (Promega, Madison, WI, USA). The structure of the HR substrate and the sequence homology, usually its sister chromatid (15,16). HR is most active in the late S and G2 phase of the cell cycle. By contrast, NHEJ is active throughout the cell cycle and requires little or no DNA homology during repair (17,18). The NHEJ pathway plays a key role in the repair of DNA DSBs caused by IR.

Cell culture and transfection. HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin and glucose. SO-Rb50 retinoblastoma cells, were established in 1991 in the State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, China. The cells were grown in DMEM supplemented with 10% FBS, penicillin/streptomycin and glucose. SO-Rb50 cells were transfected with an expression plasmid expressing WT Rb1 or an empty vector control (pcDNA3.1-Rb1 or pcDNA3.1-vector) respectively, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The positive clones were selected with 4,180 (500 μg/ml; Sigma-Aldrich, St. Louis, MO, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). HEK293 and SO-Rb50 cells in good condition were harvested, and total RNA was isolated using TRIZol Reagent (Invitrogen). RT-PCR was carried out using the one-step RT-PCR system (Takara, Dalian, China). The following primer pairs were used: for Rb1, 5'-TCTGTYTCAGGAAGAAACAACCA-3' (sense) and 5'-TATGTGCGATTACAACCTCAA-3' (antisense); for β-actin, 5'-CACCACTCTTGTTCAATGAG-3' (sense) and 5'-TACACAGCCTGGTACGAC-3' (antisense). For Rb1, RT-PCR was performed for 35 cycles each at 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min and a final extension at 72°C for 10 min. RT-PCR for β-actin was performed for 20 cycles, each with the same temperature and time parameters as for Rb1.

Immunofluorescence of cells in suspension. Non-adherent SO-Rb50 cells, SO-Rb50 cells transfected with pcDNA3.1-Rb1 and SO-Rb50 cells transfected with the pcDNA3.1-vector were smeared across a gelatin-coated slide forming a monolayer of cells. The cells were fixed in methanol and then characterized by staining with mouse anti-rat Rb1 monoclonal antibody (1:100; Wuhan Boster Biological Technology Ltd., Wuhan, China). For the negative controls, the primary antibody was replaced with PBS.

Cytogenetic techniques. For chromosome analysis, the retinoblastoma cells (SO-Rb50) at the 825th passage were used. In brief, the cells in the exponential growth phase were incubated with 40 mg/ml colchicine at 37°C for 2 h and harvested by centrifugation (1,000 rpm, 5 min). The single cells were suspended in 8 ml hypotonic solution of 0.075 mol/l KCl for 20 min at 37°C and then pre-fixed for 5 min in 1 ml of cold Carnoy's fixative (methanol:acetic acid, 3:1) by centrifugation (1,000 rpm, 5 min). The cell pellets were fixed in 8 ml cold Carnoy's fixative for 15 min. Following centrifugation, the cells were resuspended in 8 ml Carnoy's fixative at 4°C overnight. Slides were prepared using the conventional drop-splash technique [Lucas et al (19)] and then incubated at 70°C for 2 h. Slides were incubated in the trypsin solution for 75 sec and then stained with 5% Giemsa for 10 min. Fifty-six photographed cells at metaphase on the slides were counted under an Olympus BX40 microscope, and the chromosome karyotype was analyzed according to the ‘International System for Human Cytogenetic Nomenclature’ (ISCN 1978).

Assay of DNA repair efficiency in vitro. To analyze the DNA repair efficiency of exogenous Rb1, SO-Rb50 cells transfected with the pcDNA3.1-Rb1 or pcDNA3.1-vector were exposed to IR [137Cs (dose rate, 0.67 Gy/min)]. Following incubation for 0, 2.5, 8 and 24 h, the cells were smeared across a gelatin-coated slide forming a monolayer of cells. The cells were then fixed with rabbit monoclonal anti-phospho-H2AX ser-139 antibody (Millipore, Billerica, MA, USA). The secondary antibody was anti-rabbit Alexa Fluor 546-conjugated antibody (Invitrogen). Total cells were counted under a fluorescent microscope (100 objective; Carl Zeiss, Göttingen, Germany), and cells containing >10 foci were scored as positive. At least 500 cells were counted. All experiments were repeated up to four times. Error bars shown are standard errors of the mean of at least three independent experiments.

Assay of NHEJ and HR by circularization of linear plasmid substrate in SO-Rb50 cells. The experimental strategy for the NHEJ assay is depicted in Fig. 3A and B. To examine the efficiency of NHEJ or HR in the SO-Rb50 cells, the cells were transfected with the linearized pEGFP-N1 or pEGFP-HR plasmids digested with EcoR1 or Bcgl, respectively. If NHEJ or HR
occurred, the normal expression of GFP could be observed. The intact pEFGP-N1 was used as the positive control and treatment with PBS with no plasmid was used as the negative control. Forty-eight hours later, the cells were harvested and subjected to two-color fluorescence analysis. The green fluorescent cells represented the repaired DSBs and the restoration of GFP expression. The red fluorescent cells represented exogenous DNA transfection efficiency. For each analysis, 200,000 cells were processed. The relative NHEJ and HR rejoining activity was obtained by the ratio of green to red fluorescent cells.

**Cell viability assay (MTT).** The viability of the SO-Rb50 cells transfected with the pcDNA3.1-Rb1 or pcDNA3.1-vector was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) assay at three weeks following G418 selection. A total of 500 cells was seeded in 48-well culture plates. Cell proliferation was determined using the MTT Cell Proliferation Assay kit (American Type Culture Collection, Manassas, VA, USA) according to the manufacturer’s instructions.

**Cell cycle assay.** The SO-Rb50 cells transfected with the pcDNA3.1-Rb1 or pcDNA3.1-vector were harvested, fixed with 75% ice-cold ethanol in PBS and kept at 4°C. Prior to analysis, the cells were washed twice with PBS and then incubated for 30 min in a propidium iodide staining solution containing 0.05 mg/ml propidium iodide, 1 mM ethylene-diaminetetraacetic acid (EDTA), 0.1% Triton X-100™ and 1 mg/ml ribonuclease A (RNase A) (all from Sigma-Aldrich). The staining fluorescence intensity was measured using a BD FACSort™ flow cytometer (BD Biosciences, San Jose, CA, USA) and used to determine the G2/M ratio.

**Statistical analysis.** Data shown are representative of three independent experiments with each experiment performed in triplicate. Data are expressed as the means ± SD. Statistical analyses were performed using the SPSS for Windows version 10.5 software package. The differences between mean values were evaluated using the two-tailed Student's t-test (for two groups). A P-value <0.05 was considered to indicate a statistically significant difference.

**Results**

**Rb1 gene mutation and DNA instability in SO-Rb50 cells.** As shown in previous studies, the Rb1 gene is mutated in SO-Rb50 retinoblastoma cells (20). Our data confirmed that there was a mutation with a primer pair located in exon 14 and 17 by RT-PCR analysis (Fig. 1A). The results of immunofluorescence staining also showed that the Rb1 protein is not expressed in SO-Rb50 cells (Fig. 1C).

In order to confirm the data from previous studies showing SO-Rb50 cells display obviously chromosomal instability (8), we performed karyotype analysis of 825th passage SO-Rb50 cells. Our data revealed both numerical and structural chromosomal aberrations in the SO-Rb50 cells. The chromosome assay showed that the number of chromosomes in the cells ranged from 22 to 93. The majority of the cells (47%) had chromosome numbers of ≥46 and the metaphase spread with a normal diploid number (2N=46) was approximately 30%. The cells with chromosome numbers of >46 accounted for 23%. The type of chromosomal aberrations included chromosome breakage, shift, rearrangement, deletion, repeat, etc. The variation of several chromosomes was so severe that they could not be recognized (Fig. 1D and E). Polyploid cells could also be observed occasionally (Fig. 1B).

**Rb1 does not affect the repair of DNA DSBs following exposure to IR.** In order to elucidate the mechanism behind the chromosomal instability of retinoblastoma cells, we first investigated the effect of Rb1 on the repair of DNA DSBs induced by exposure to IR. The SO-Rb50 cells were transfected with the pcDNA3.1-Rb1 plasmid, expressing WT Rb1 or the pcDNA3.1-vector. Fig. 2A shows that exogenous Rb1 is highly expressed in the cytoplasm of SO-Rb50 cells following transfection with the pcDNA3.1-Rb1 plasmid. Following exposure to 0, 2.5 or 5 Gy IR, the SO-Rb50 cells with and without exogenous Rb1 were fixed at different time points, 0, 2.5, 8 and 24 h post-damage, and stained with γ-H2AX, a commonly used in situ marker of DNA DSBs. Our data revealed that there was a significant increase in γ-H2AX expression following exposure to 2.5 or 5 Gy IR compared with the controls (0 Gy) (set to 100%) in the SO-Rb50 cells transfected with the pcDNA3.1-Rb1 or pcDNA3.1-vector during the first 8 h. However, exogenous Rb1 did not affect γ-H2AX expression, as shown by images of the cell population taken following exposure to equal doses of IR (P>0.05) (Fig. 2D). These results are in agreement with those from a previous study (14). These data suggest that Rb1 does not affect the repair of DNA DSBs following exposure to IR.

**Exogenous Rb1 does not affect NHEJ, but promotes HR of SO-Rb50 cells.** A previous study reported that NHEJ is a rapid process, which can be completed in approximately 30 min, while HR is much slower and takes 7 h or longer to complete (21). Moreover, the repair of IR-induced DNA DSBs is catalyzed predominantly by the NHEJ pathway (22). Therefore, we further wished to assess NHEJ and HR activity, separately, in SO-Rb50 cells, as described in Materials and methods. For NHEJ assay, DNA substrate with either complementary ends was prepared by linearizing pEGFP-N1 with EcoRI. The cleavage between the promoter and the GFP reporter gene thereby prevents the expression of the reporter in vivo (Fig. 3A). Intracellular recircularization of the linearized DNA through NHEJ repair-mediated end rejoining allows for the expression of GFP, which was then assayed by flow cytometry analysis. The rejoining levels in the cells revealed that exogenous Rb1 had no differential effect on NHEJ (Fig. 3C).

For HR assay, we constructed the recombination substrate, pEFGP-HR, which contains two GFP cDNA fragments after the promoter, GFP1 in +1 to +400 bp; GFP2 covers the whole cDNA. There is an inserter between GFP1 and GFP2, which results in the abnormal expression of GFP. The DNA substrate with HR ends was prepared by linearizing pEFGP-HR with BglII. If HR occurs, GFP is expressed by sharing extensive sequence homology (Fig. 3B). As shown in Fig. 3D, the level of HR in the cells expressing exogenous Rb1 was significantly enhanced by 2.46-fold compared with the control cells (P<0.01). These findings provide direct evidence that Rb1 enhances HR, but does not affect NHEJ.
Exogenous Rb1 affects cell viability and the cell cycle of SO-Rb50 cells in vitro. HR is most active during the late S and G2 phase of the cell cycle. Therefore, we examined the cell cycle of SO-Rb50 cells with and without exogenous Rb1. The results of MTT assay revealed that the viability of SO-Rb50 cells transfected with pcDNA3.1-Rb1 was reduced by 17.46±2.66% compared with the control group, which demonstrated that exogenous Rb1 significantly inhibited the growth of SO-Rb50 cells (P<0.01) (Fig. 4A). Flow cytometric analysis revealed that, compared with the control group, the percentage of SO-Rb50 cells with exogenous Rb1 in the G0/G1 phase and G2/M phase was decreased from 41.85±4.30 to 35.69±1.54% (t=3.665, P<0.01), and from 25.23±2.77 to 21.23±4.00% (t=2.251, P<0.05) respectively, and that in the S phase was increased from 32.92±1.48 to 43.08±2.23% (t=10.396, P<0.001) (Fig. 4B). These results indicate that exogenous Rb1 promotes the arrest of cells in the S phase of the cell cycle, thereby inhibiting SO-Rb50 cell proliferation.
Discussion

A growing body of evidence suggests that the genomic instability of retinoblastoma is observed both in vitro and in vivo (9,10,23). However, the effect of Rb1 on the DNA DSB repair process remains unclear. Whether the particular DNA DSB repair sub-pathway is controlled by Rb1 is also unknown. To address these issues, we first verified the loss of Rb1 in SO-Rb50 retinoblastoma cells. Our results indicated that there was a mutation with a primer pair located in exon 14 and 17 by RT-PCR (Fig. 1A). The staining of Rb1 confirmed that Rb1 was not expressed in the SO-Rb50 cells (Fig. 1C). These data are consistent with those from a previous report (24).

Moreover, we further demonstrated the genomic instability of SO-Rb50 cells by karyotype analysis. As shown in a previous study, Feng et al (9) screened the promoter and 27 exons of the Rb1 gene in SO-Rb50 cells using polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) and southern blot analysis at different passages. They found new mutation events that occurred in exons 23, 24 and 25 in consecutive passages, compared to the 451st passage. G-banding and karyotype analysis further proved that there were chromosomal aberrations, which were observed in the same passage of different cell strains of SO-Rb50 cells. We analyzed the SO-Rb50 cells at the 825th passage. Our data showed that 47% of the SO-Rb50 cells displayed both numerical and structural chromosomal aberrations. Only approximately 30% of the cells had a normal diploid number (2N=46). The heteromorphosis of several chromosomes was too severe to be recognized (Fig. 1B, D and E). These results strongly suggest that the mutation of Rb1 causes dynamic chromosomal alterations during long-term culture in vitro.

Similarly, a study on sporadic unilateral retinoblastoma tumors performed by Ganguly et al (23) indicated that tumors harbored novel regions of amplification at 1q44, 3p25, 11q14, 11q25, 14q23, 15q21, 16p13, 17p11.2, 19q13 and 20q13, while regions of loss included 6q22, 7q21 and 21q2.

DNA DSB repair plays a key role in genomic stability. IR-induced damage can result in DNA DSBs in cells. Therefore, we first examined whether exogenous Rb1 affects the repair of DNA DSBs induced by IR in SO-Rb50 cells. The stable cells expressing WT Rb1 and the control cells were treated with 2.5 and 5 Gy radiation. At different time points, the cells were fixed and stained with γ-H2AX. The formation of γ-H2AX foci in the cells transfected with the pcDNA3.1-Rb1 or pcDNA3.1 vector following exposure to IR at different time points. Data (means ± SEM from three independent experiments) show the percentage of cells containing >10 foci.
formation in cells, we found that IR treatment significantly induced DNA DSBs in SO-Rb50 cells both with and without exogenous Rb1. However, exogenous Rb1 had no significant effects upon γ-H2AX foci intensity, as shown by images of the cell population taken at following exposure to equal doses of IR. Both cells with WT Rb1 and the empty vector displayed a similar increase in staining intensity during the first 8 h post-damage and a similar kinetic decrease in intensity from 8 to 24 h (Fig. 2D). These results are in agreement with those from a previous study (14), which indicated that Rb1 had no significant effects on direct DNA repair following IR-induced damage in SO-Rb50 cells. Moreover, it is well established that NHEJ is the main pathway for the repair of the majority of IR-induced DNA DSBs throughout the cell cycle (27-30). Our results suggest that Rb1 does not affect the NHEJ sub-pathway.

As described as above, DNA DSB repair involves NHEJ and HR. The error-prone NHEJ pathway rapidly and promiscuously rejoins the ends of broken chromosomes while HR repair uses a homologous template in a sister chromatid or homologous chromosome to perform error-free repair (31-33). Rb1 does not affect NHEJ. Therefore, we hypothesized that the chromosomal aberration in SO-Rb50 Rb1-deficient cells is induced by preventing error-free HR. In order to demonstrate this hypothesis, we used recircularization assay with the linearized pEGFP-N1 and pEGFP-HR substrate to examine the role of Rb1 in NHEJ and HR. Consistent with our results on IR-induced damage (Fig. 2D), Rb1 did not affect NHEJ (Fig. 3C). Conversely, in the presence of WT Rb1, the level of HR in the cells expressing exogenous Rb1 was significantly enhanced by 2.46-fold (P<0.01 vs. control).
the deficient HR may result in chromosomal instability and chromosomal aberration in SO-Rb50 cells. HR is most active during the late S and G2 phases of the cell cycle. Therefore, we investigated the effect of Rb1 on the cell cycle and viability of SO-Rb50 cells. Our results revealed that WT Rb1 reduced the viability of SO-Rb50 cells and significantly promoted the arrest of cells in the S phase of the cell cycle (Fig. 4), thereby inhibiting SO-Rb50 cell proliferation. Our results are consistent with those from previous studies, showing that Rb1 regulates the cell cycle, differentiation, growth and apoptosis (5-7). It is now clear that the complex of Rb1 binding transcript factor, E2F, plays a crucial role in regulating the initiation of DNA replication. A number of previous studies have demonstrated that E2F regulates many downstream target genes that are involved in cell cycle progression and DNA replication, such as cyclin A, cyclin E, cdc2 and cdk2, proliferating cell nuclear antigen (PCNA), mini-chromosome maintenance-7 (MCM-7), topoisomerase IIa and thymidine kinase (34,35). It is possible that the loss of Rb1, which occurs concomitantly with the vast target gene deregulation, facilitates the bypass of the cell cycle checkpoint, which is one of the mechanisms by which a tumor can occur.

In conclusion, this study provides evidence that chromosomal aberrations occur in Rb1-deficient SO-Rb50 retinoblastoma cells. The assay of DNA DSB repair demonstrated that Rb1 does not affect NHEJ and significantly promotes HR. To our knowledge, this is the first study revealing the mechanism of action of Rb1, namely the regulation of the sub-pathway of DNA DSB repair. An in depth understanding of the mechanism by which the Rb1 tumor suppressor gene regulates the growth of tumor cells may provide us with valuable information for the development of novel methods of therapeutic intervention and treatment against retinoblastoma tumors.

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References